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Life History Patterns of *Syngnathus typhle*

An Experimental Approach

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Diploma Thesis

Abstract

Syngnathus typhle disappears during wintertime from the eelgrass meadows, as temperatures, currents and leave shortage during winter is not favorable for pipefish. Migration patterns were revealed by otolith microchemistry and stable isotope analysis of muscle tissue. Otolith microchemistry was experimentally evaluated, to disentangle the relationships of environmental conditions and the Sr incorporation in the otolith. The incorporation was dependent on salinity, otolith growths and at low salinities on somatic growth. Temperature had only an effect on otolith growth, and therefore an indirect effect on the Sr/Ca-ratio. Patterns associated to migrations could not be found in the otolith. Further a food shift related to migration was not detected, as the stable isotope values followed a general more pelagic pattern throughout the year. For the time in the eelgrass meadow niche theory was tested with the comparison to two other Syngnathids (*Syngnathus rostellatus, Nerophis lombriciformi*), where a strong niche formation could be observed. For *S. typhle* and *N. lmobriciformi* a change of trophic level with size was shown, while *S. rostellatus* had a broad range of trophic levels which suggests a more general feeding behavior compared to the others.

Zusammenfassung

Im Winter ist die Grasnadel (*Syngnathus typhle*) nicht in den Seegraswiesen anzutreffen, da die Kälte, durch Wind verursachte Turbulenzen und das weitestgehend reduziertes Seegras keinen optimalen Lebensraum mehr darstellen. Anhand von Otolithen Microchemie und der Analyse stabiler Isotope aus Muskelfleisch wurden Migrationshinweise untersucht. Durch ein Experiment wurden die Umwelteinflüsse entschlüsselt, welche einen Einfluss auf die Strontium Einlagerung im Otolithen haben. Die Strontium Einlagerung war abhängig von Salinität und Otolithenwachstum und bei niedrigen Salinitäten auch vom Wachstum des Tieres. Temperatur hatte nur über das Otolithenwachstum einen indirekten Effekt. In den Otolithen konnten keine Hinweise gefunden werden, die es erlauben das Migrationsverhalten zu rekonstruieren. Ein migrationsbedingter Futterwechsel konnte ebenfalls nicht gezeigt werden, da eine ohnehin stark pelagisch geprägte Ernährung anzunehmen ist. Für die Sommerzeit konnte die Nischentheorie mittels eines Vergleichs zu zwei anderen Syngnathiden (*Syngnathus rostellatus, Nerophis lombriciformi*) bestätigt werden. Weiter wurde für *S. typhle* und *N. lmobriciformi* eine Zunahme der trophischen Ebene mit zunehmender Größe gezeigt, wohingegen *S. rostellatus* innerhalb einer Größenordnung variable trophische Ebenen einzunehmen schien, was auf ein undifferenziertes Fressverhalten hindeutet.

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General Introduction

Seasonal change is common in temperate zone which influences ecosystems and their residents. Often these ecosystems represent habitats for specific time frames and species, as they become unsuitable during wintertime. To avoid these conditions animals regularly migrate, causing annual migration patterns, which were studied at Syngnathids and the ecosystem eelgrass meadows in this thesis.

In the eelgrass meadow only a limited number of animals feed directly on eelgrass, but epifauna and microalgae coverage on the leaves provide a food source for many invertebrates which then build a base for higher trophic levels (Duffy et al. 2003; Thayer et al. 1975). The eelgrass leaves retard currents, regulate nutrients and oxygen balance, improve water quality by filtering suspended matter and the rhizome system stabilizes bottom sediments (Short and Neckles 1999), what makes the eelgrass meadow a perfect nursery habitat for commercial important and other fish (Bostrom et al. 2006; Duffy et al. 2003; Thayer et al. 1975; Verweij et al. 2008).

Annual variations in eelgrass growth and a leave life-time of less than a year due to autumn storms and current, lead the eelgrass meadows almost disappear during wintertime (Duarte et al. 1994). This hinders animals to hide in the shelter of eelgrass, decreases important food sources and thus forces many fish species to migrate from the eelgrass ground, so do pipefish (Vincent et al. 1995). However, where they spend their winters in northern European regions so far remains a mystery.

The prominent reason for migration of fish seem to be reproduction, as the movements primarily occur between different feeding and spawning grounds, which is strikingly visible for diadromous species like eel, trout and salmon (Daverat et al. 2006; Godbout et al. 2010; Leggett 1977; Quinn et al. 2007; Zurstadt and Stephan 2004), but has also been suggested to be reason for oceanic migration of bluefin tuna (Block et al. 2005). In case of Syngnathids mating and feeding occurs in the eelgrass meadows, and therefore reproduction aspects seem to play a different role for migration.

The mate season for pipefish lasts from May to September, which roughly fits the time pipefish occur in the eelgrass meadows (Vincent et al. 1995). During mating eggs are transferred from the female to the brooch pouch of the male where embryos develop

for 4-6 weeks, depending on the temperature (Ahnesjö 1995). To accelerate the development of the embryos, pregnant male pipefish swim to warmer waters and can thus be found in more shallow waters than females (Vincent et al. 1995).

Genetic exchange between northern Syngnathids populations have been shown by population analyses based on neutral microsatellite markers, which show no population differentiation within northern pipefish population (Wilson and Eigenmann Veraguth, 2010; Roth et al. 2012, Evolution accepted). As pipefish are born in the eelgrass meadows and grow there during the summer season, mixing of populations possibly takes place during wintertime.

Syngnathus fuscus, a pipefish species in north America, migrates in autumn offshore to the continental shelf into depth down to 250 m and comes back to the eelgrass meadows in spring (Lazzaril and Able 1990). Lazzaril and Able suggest the migration to be temperature triggered. The distance can be up to several hundred kilometers.

In the western Baltic *Syngnathus typhle*, *Syngnathus rostellatus, Nerophis lombriciformi, Entelurus aequoreus* and *Syngnathus acus* are representing this scientific family (Vincent et al. 1995), and so far the migration behavior and ecologic aspects have fairly been described (Martin-Smith 2006).

Different Syngnathids may fulfill different ecological niches within the eelgrass meadows, as the dietary preferences of pipefish are partly known and may be determined by their size and snout form. While the worm pipefish *N. lombriciformi* and *E. aequoreus* with short snout feed on prey hidden in the vegetation, *S. typhle* with a long and high snout feeds on large and fast pelagic prey (Franzoi et al. 2004). Sympatric species within one habitat tend to fulfill different ecological niches (Friberg et al. 2007). The concept of ecological niches described by Hutchinson (1957), although the phenomenon of different ecological niches of finches already leads Darwin to his conclusions about evolution.

The ecological niche does not have to stay the same during the whole life. Many teleost fish start their life as planktivorous larvae and feed as adults on other fish. Gut content analysis of *S. typhle* show a food shift during lifetime. Juveniles feed mainly on

copepods, whereas medium sized specimens feed mainly on Hippolytidae and Mysidacea a, while larger specimens prey on Hippolytidae, Palaemonidae and Gobiidae (Oliveira et al. 2007). Prey size generally increase with size of *S. typhle*.

Aims of this study

This aim of this study was to investigate migration patterns and of *Syngnathus typhle* and niche formation within the Syngnathids. As migration patterns of small animals can still not be followed by satellite tags, here two different passive approaches were used to search for fingerprints of migratory behavior of *S. typhle.* An experimental approach was used to evaluate otolith microchemistry and further the gained information was used to analyze otoliths of wild caught fish.

For large scale migration patterns of salinity change in the otoliths were expected since the Baltic is a quite diverse habitat with several salinity gradients, vertical, as saltier water is denser and therefore is found in deeper water layers, and horizontal, as the salinity declines in the surface waters with increasing distance to the North Sea.

Along a different line stable isotope analysis of muscle tissue was used to identify a potential switch of food sources. The analysis of those fingerprints may give us insight into the life of pipefish during wintertime.

Further niche theory was tested upon three different pipefish species (*S. typhle*, *S. rostellatus*, *N. lombriciformi*) using stable isotopes of muscle tissue. This was going to expand the knowledge about life history of Syngnathids.

Chapter 1: Migration of *Syngnathus typhle* based on Otolith Microchemistry

Introduction into Otolith Microchemistry

Otoliths are part of the vestibular apparatus, which teleost fish need for their balance and hearing (Fig. 1). They are pair wise structures made of calcium carbonate. Otoliths can be classified into three types: Sagitta, lapillus and asteriscus (Fig. 2). Sagitta and lapillus usually consist of aragonite causing a milky appearance while asteriscus often consists of vaterite what is responsible for its clear look (Thresher 1999). Density of the otoliths is higher compared to the fish and to the surrounding seawater, hence the otoliths move with different amplitude (Popper et al. 2005). Microvilli of sensory cells touching the otoliths sensing the mechanical signal (Popper et al. 2005). Popper et al. also suggest an influence of size and shape of the otoliths on the hearing capabilities.

The initiation of otolith growth is the nucleation. Several protein nuclei initiate the accumulation of calcium carbonate (Payan et al. 2004). Calcium carbonate is typically accumulated with daily variation as daily fluctuations influences otolith growth (Panella 1971). These layers can be optically distinguished via light microscopy (Panella 1971). In fish older than a year, daily rings become more difficult to distinguish while yearly variations turn to be easier to differentiate by refined methods (Brothers et al. 1975;

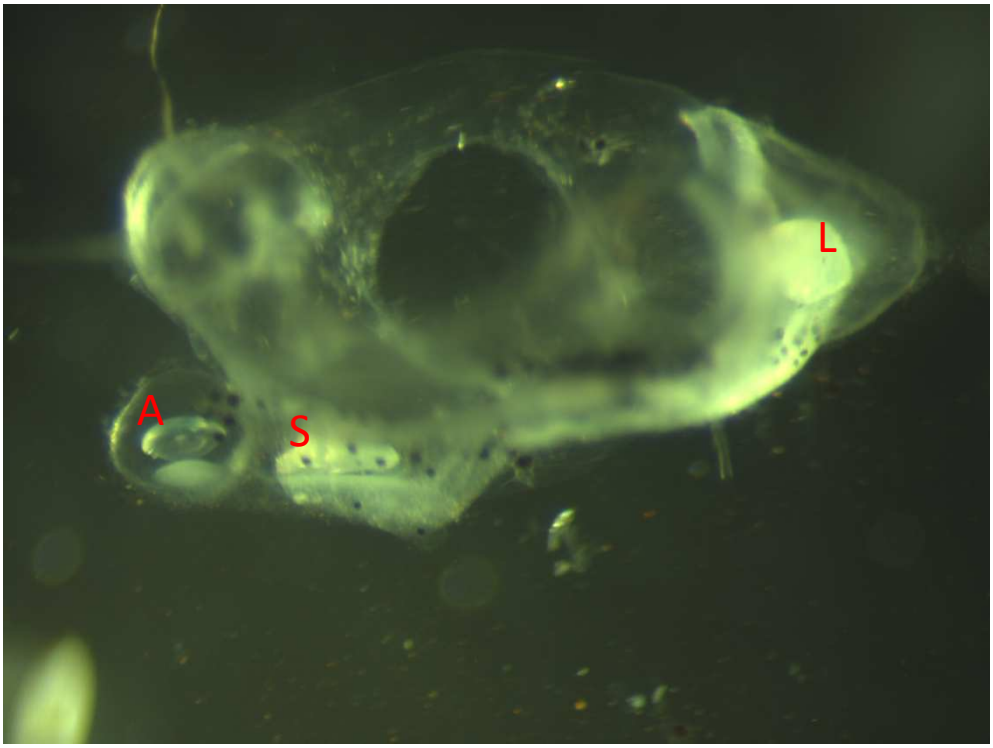

Fig. 1 Vestibular apparatus of *Syngnathus typhle*. A asteriscus, S sagitta, L lapillus

Cermeno et al. 2003; Christensen 1964). However, in old fish even those yearly variations become difficult to differentiate as otolith growth reduce with age (Campana 2001). The visible ring structure is comparable to the ring structure of a tree. In Syngnathids the ring structure of otoliths seems to be independent of daily and annual growth variations (as shown for *Hippocampus spinosissimus*), what makes exact age determination difficult (Do et al. 2006).

More life history information is available through microchemical analysis of the otoliths. As otoliths grow continuously over the entire life time of a fish and are assumed to be chemically inert, each layer of calcium carbonate stores information about the water properties at the time it was precipitated (Thresher 1999). The chemical composition depends on various parameters: physical such as water temperature and salinity, also metabolic rate (synthesis of matrix protein, e.g. otolin), and even a correlation between element/Ca-ratios and genetic factors has been reported (Clarke et al. 2011; Thresher 1999). The main component of aragonite is calcium carbonate (95%) whereas only four percent are of organic material (Campana 1999). Minor and trace elements make up to less than one percent (Campana 1999). Aragonite is calcified into a protein matrix, which mediates or probably controls the calcification process (Murayama et al. 2002).

The chemical composition of otoliths is not directly proportional to the composition of the water in which the fish lives although the majority of inorganic elements derived from the water (Campana 1999; Payan et al. 2004). Several barriers have to be passed by the elements on their way from the water to the otoliths. Each barrier in general describes one potential modulatory device. For marine animals the first barrier is the intestine where the elements are assimilated and transferred into the blood plasma (Campana 1999). For fresh water the first barrier constitutes the gills, due to their different osmoregulatory requirements (Campana 1999). The secretion of the

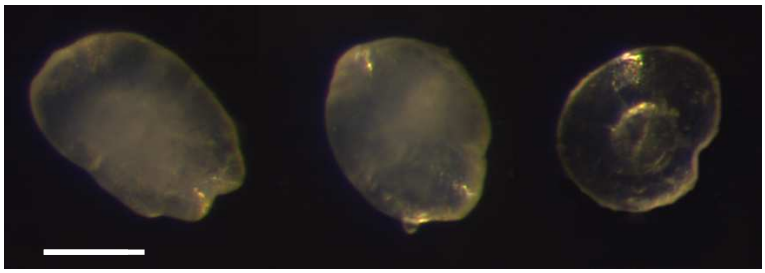

Fig. 2 Otoliths of *Syngnathus typhle*. From left to right: sagitta, lapillus, asteriscus. Bar is 100µm.

endolymph at the inner-ear epithelium and finally the crystallization itself display further barriers (Payan et al. 2004).

Among others electron microprobe analysis (EMPA) provides a widely used method for otolith microchemical analysis. EMPA is used to quantify higher concentrated trace elements (>100 ppm) at a very high spatial resolution (1µm^2). The comparable low size of pipefish otoliths requires high spatial resolution of the measure method. For that reason the electron microprobe was chosen as analytical procedure. EMPA is based on different rebound angles of x-rays when electrons hit on atoms of certain elements. Therefore the sample is in a vacuum chamber and is shot with an electron ray. Detectors positioned in the specific rebound angle for the searched element count the reflected electrons.

Widely used to reconstruct migration patterns is the strontium (Sr) – calcium (Ca) – ratio (Sr/Ca) (Elsdon and Gillanders 2003). The strontium incorporation is environmentally influenced and its concentration is comparably high to other elements and therefore easy to detect (Elsdon and Gillanders 2003). Other trace elements like sodium (Na), chlorine (Cl), zinc (Zn) and potassium (K) are easily leached out, although their concentrations would be high enough to be detected (Campana 1999). Beside that those elements are highly regulated in the blood plasma and therefore inappropriate to serve as environmental tags (Elsdon and Gillanders 2003).

General accepted is the correlation of surrounding water salinity and Sr/Ca-ratios of otoliths. Water Sr concentrations usually increase with salinity. Otolith Sr/Ca-ratios were therefore successfully used to reconstruct migrations between marine and freshwater habitats (Albuquerque et al.2010; Daverat et al. 2006).

Salinity is not the only parameter influencing the Sr/Ca-ratio. Also temperature is considered to have a significant effect on Sr/Ca. Reason for this might be the kinetic effects described in the equation Kinsmann and Holland published in 1969 for aragonite crystallization (Campana 1999): Sr/Ca mmol mol^{-1} = 10.66 – 0.039 x temperature. This equation is not transferrable to otolith formation, as the equation only covers inorganic aragonite formation but not biological processes. Temperature as influencing factor is controversially discussed. Although positive correlations have been reported, also

negative and non-existent results have been published throughout the years (Campana 1999). Annual variations are not seldom, which often fit with the temperature regime of the region, but are not necessarily correlated with temperature (Campana 1999). Sadovy and Severin (1994) discussed the temperature effect as artificial, because Sr/Ca-ratios would only reflect growth rates, which are related to temperature. A tendency towards lower Sa/Ca-ratio for faster growing individuals had been observed in their studies. Further an increase for the Sr/Ca-ratio with age has been reported (Proctor et al. 1995; Radtke 1987; Radtke and Targett 1984; Sadovy and Severin 1994).

The environmental effect on the Sr/Ca-ratio is species specific (Campana 1999). Therefore the dependence on environmental factors has to be determined for every species before interpreting microchemical patterns (Elsdon and Gillanders 2003).

Besides the interpretation of patterns of naturally formed Sr/Ca marks in otoliths can also be marked with trace elements (Campana 1999). Aside from trace elements chemical markers like alizarin which can be optically distinguished via light microscopy (Campana 1999), chemical- and trace elements marks are often suggested to be used as mass marker for fish stocks (Campana 1999; Kuroki et al. 2010; Thorrold et al. 2006; Woodcock et al. 2010). For this study a chemical mark will be used as marking for the experimental start.

Preview Study on *Syngnathus typhle* Otoliths

Baseline for this study is the previous work of Miersch *et al.* (unpublished). Pipefish otoliths for patterns in Sr were scanned. Indeed a common pattern of two Sr-rings could be found, which were suggested to be related to migration in winter (Fig. 3). The present study aims at clarifying whether these rings are correlated to migratory behavior or not.

To investigate how these rings were formed and if the pattern is dependent on environmental factors, the influence of two environmental parameters (salinity and temperature) on the Sr/Ca-ratio will be experimentally assessed. In addition, the gained information will be used to classify the whole otolith patterns of wild-caught fish.

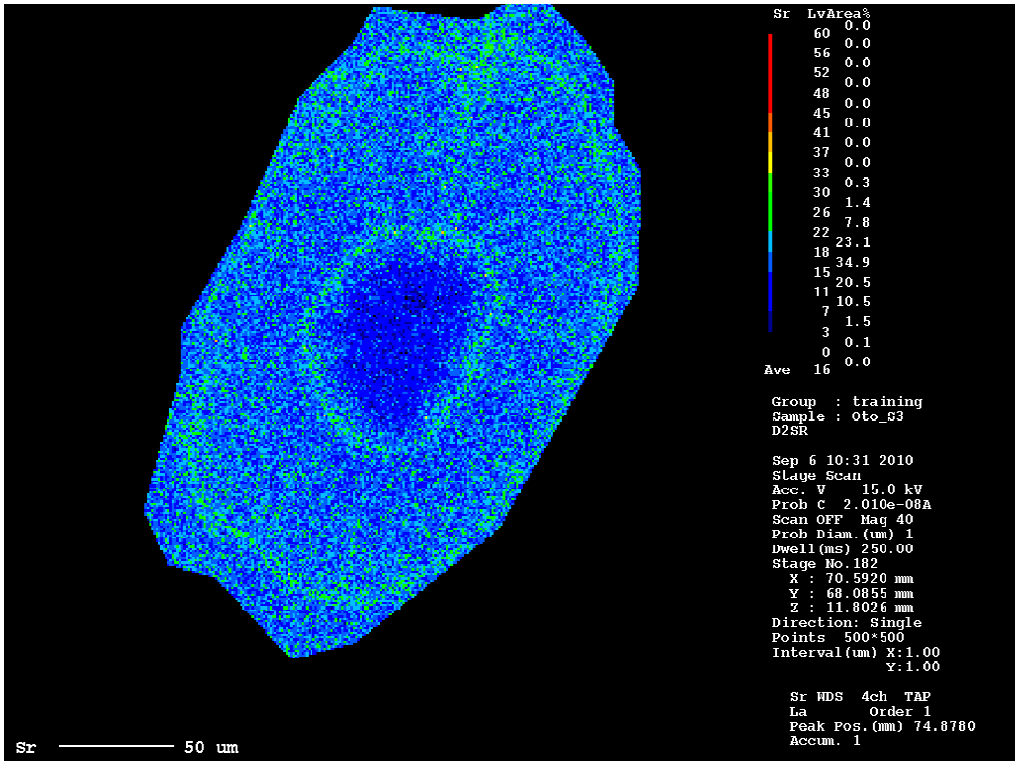

Fig. 3 Electrone microprobe mapping picture of a *Syngnathus typhle* otolith. Two Sr-rings are clearly visible. One close to the core and another further distal.

Material and Methods

Animal Capture and Treatment for Experiment

120 animals for the experimental part were captured at Gelting Bay at the 17.06.2011. Animals were caught with handnets during snorkeling. After the transport to the Institute the animals were immediately put into a strontium (Sr) ($SrCl_2$, Merck) enriched (ca. 44 g $SrCl_2$/200 L water from Kiel Fjord (salinity: 16)) aquarium tank. This work step led to an enrichment of Sr in the otoliths and hence provides a mark that labels the start of our experiment. The animals were exposed to the higher Sr-conditions for three days. After the initial marking with Sr of the otoliths the animals were washed in Baltic Sea water to not introduce artificial Sr into to the experimental facility. Before the start of the experiment all animals were measured (total length) and tagged with an individual three color code (color1 (treatment)/ color2 (aquarium)/ color3 (individual)). For tagging visible implant elastomeres were injected under the skin (Northwest Marine Technology, Inc.). All males (n=94) used in the experiment were pregnant at the beginning of the experiment. Juveniles born during the experiment were removed and raised up separately at an approximate salinity of 17. Otoliths were later on taken from two of them.

Animal Capture and Treatment for Wild Caught Fish Otoliths

Animals for whole otolith analysis were collected during May and June 2011. The *Zostera marina* meadows the animals were taken from are located at Doverodde (Denmark; +56° 43' 8.37", +8° 28' 24.53"; N=9), Fiskebäckskil (Sweden; +58° 15' 2.31", +11° 27' 2.83"; N=14), Gelting Bay (Germany; +54° 45' 29.57", +9° 52' 34.25"; N=6) and Kiel Fjord (Germany; +54° 26' 4.23", +10° 10' 12.10"; N=25) (Fig. 4). After they were captured the animals were held in aquaria at salinity of 16 for up to two months. Too prevent the analysis of material grown under artificial conditions, the outer edge of these otoliths was not considered for analysis. No length data were available for these individuals. The otoliths of non experimental animals are labeled as field sample otoliths.

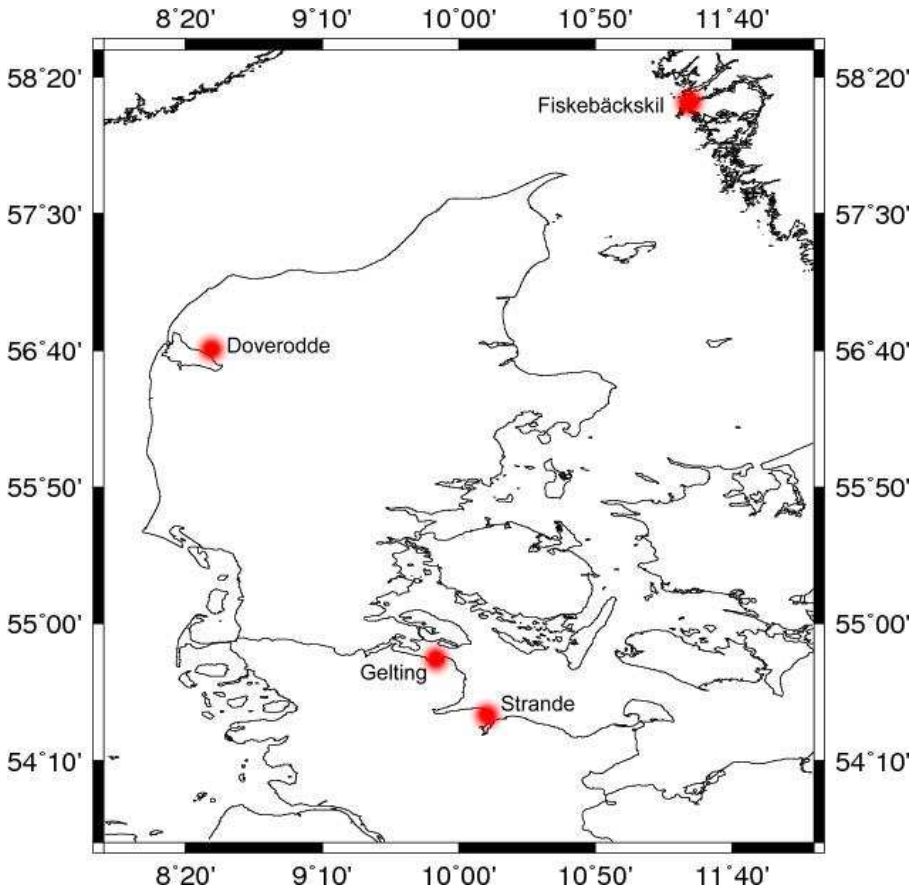

Fig. 4 Sample sites in the Western Baltic. Animals used in the experiment derive from Gelting. At the other sampling locations only animals for whole otolith analysis were sampled. (Graphic from: NOAA Coastline Extractor)

The Experimental Setup

The aim of the experiment was to investigate salinity and temperature dependence of strontium incorporation into *S. typhle* otoliths. For this, three salinities were chosen: 15, the actual salinity of the Kiel Fjord; 23, average Kattegat salinity; and 30, salinity in the Skagerrak (Schramm 1996). As cold temperature 12°C was chosen and 20°C as warm (Tab. 1). These are temperatures pipefish regularly experience in the eelgrass meadows (Berglund et al. 1986; Riccato et al. 2003). The experimental design thus resulted in six treatments. For each treatment one circular system was built, containing four aquaria, one biofilter, UV-filter, and skimmer (Fig. 5). The seawater used for the experiment was obtained by the FS Alkor. The water was taken north-west of Helgoland (salinity 32) and carried in the ballast tank of the ship. Water was thereafter stores in plastic tanks (1.5 m^3 each, N=6). The tanks were protected by a canvas cover against rainfall and sunlight

and the water in the tank was permanently bubbled with air. For the experiment the seawater was diluted with tab water to reach the required salinity. The animals were fed with defrosted mysidacea twice a day. Temperature and salinity were measured five times a week. Cleaning and water exchange were done twice a week. Otoliths of animals that died during the second half of the experiment were dissected and used for analysis. Mortality during the experiment was almost exclusively due to fish jumping out of aquaria and drying out. Size of these animals could not be taken due to shrinkage.

Salinity Temperature	15	23	30
12°C	n=4x5	n=4x5	n=4x5
20°C	n=4x5	n=4x5	n=4x5

Tab. 1 Experiment arrangement. n=number 4=number of aquaria 5= number of animals per aquaria

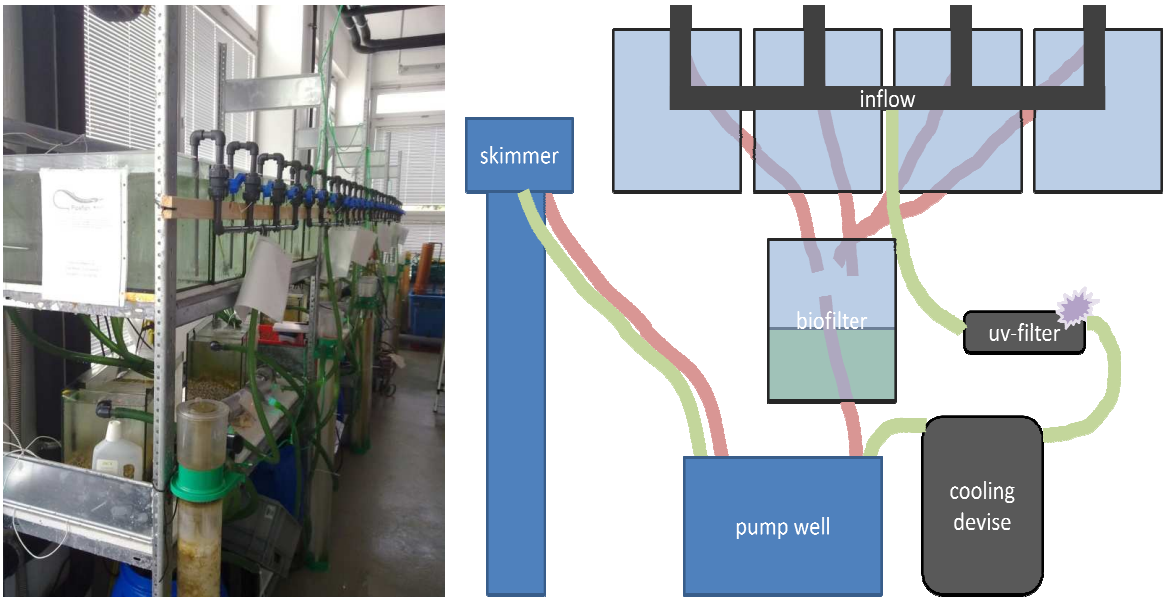

Fig. 5 Aquaria facility and Hall was conditioned and exposed to daylight. Scheme of Circulation. Green lines = inflow. Red lines = outflow. The facility contained six circulation systems.

Otolith Removal

Otolith removal was conducted under a stereo microscope. To remove the otoliths first the skullcap was removed along the dissection line shown in Fig. 6. The vestibular apparatus resides on the posterior sides (e.g. right and left) of the brain. The whole vestibular apparatus could be removed by forceps. The otoliths were dissected out of the vestibular apparatus in distilled water. The type of otolith (e.g. sagitta, lapillus, asteriscus) was determined by the shape of the otolith after Do et al., 2006. After dissection the otoliths were dried in open 500 µl Eppendorf tubes for at least 24h at room temperature. For the microchemical analysis only sagittae were used.

Preparation for EMPA

The otoliths were mounted on labeled glass slides using thermoplastic glue (Crystalbond Type 509; Kager, Dietzenbach, Germany). In this step, glass slide with glue on it was heated on a heating plate to approximately 55°C. When the glue was molted, the otolith was positioned in the glue under a stereo microscope. The plain distal side to the bottom and the convex side up, such that the otolith had contact to the glass slide and was totally covered with glue. In the next step the glass slide was grinded in order to display the core of the otolith. Optimal grinding results were achieved by placing a paper tissue on the table and position the sandpaper (0.3 µm, SIA) on it. The glass slide with the glue side down was slowly grinded over sandpaper which was wetted with distilled

Fig. 6 Head of Syngathus typhle. Red line marks dissection line.

water. During grinding the process has to be checked under the light microscope in order to hit the otolith core. When the core was reached, the otolith was finally polished with aluminum slurry (0.05 µm, Buehler), which was put on the otolith and then rubbed over with a soft towel until the otolith was totally plane. Again this work step was checked under the light microscope and repeated until there were no scratches visible on the otolith. Right before the EMPA samples were coated with carbon at 30°C to improve conductivity. In the sample chamber of the electron microprobe (JXA – 8200 WD/ED COMBINED MICROANALYZER) a pressure of 1.7 x 10^{-5} mbar was generated, close to vacuum. The accelerating voltage was 15 000 volts to get a depth of measurement of one micrometer. The voltage on the probe was 3.02e^{-8} and the intensity of the current was 40 nA. The electron ray has to be calibrated for all measured elements. To collect data the map analysis program of the EMPA was used. The electron ray and the measurement interval were set to one micrometer in diameter. The measurement speed was set to 115 ms/µm^2. The shape of the otolith was entered into the program. In order to save time only ¾ of the otolith was mapped. Each map consists of 200x200 data points. Before and after each session, standards (Calcite, KAN1, Strontianite) were measured with the same set up to calibrate the raw counts to parts per million.

Raw Data Analysis of Experimental Otoliths

To improve data quality the raw counts were interpolated using Microsoft Excel spreadsheets. Each calculated point consists of the average of nine measured data points (the point itself and the eight surrounding ones). By this method the background noise is reduced as well as spatial resolution, while accuracy for each measured point increases. For further calculations the average calibration curve for each session was determined. Each calculated point was converted from raw count to ppm. Those values for Sr and Ca were set into ratio and for better handling multiplied with 100. For easy reading only the term Sr/Ca-ratio was used although the exact nomenclature is Sr/Ca-ratio x 100.

For the experimental otoliths the material precipitated after the Sr-marking was of interest. The data were transformed with R (v. 2.13.2). The map was cut into four equal

pieces. In each piece the maximal value for each column were determined (e.g. the initial Sr-marking) and wrote every 20 values above and beneath the maximal value in a new matrix.

For row vise analysis the initial data were transposed. Each new matrix consists of a pre- and after Sr-marking region. Values at the edge of the measurement were under special concern, as the values become doubtful if the electron ray hits the glue. Therefore values at the edge which were unlikely were deleted. The unlikeliness was defined by a value smaller zero and the difference to the neighbor values of larger than 0.6. Later the averages of each row were linearly analyzed as seen in Fig. 7. Each value for an individual is the average of several measurement points.

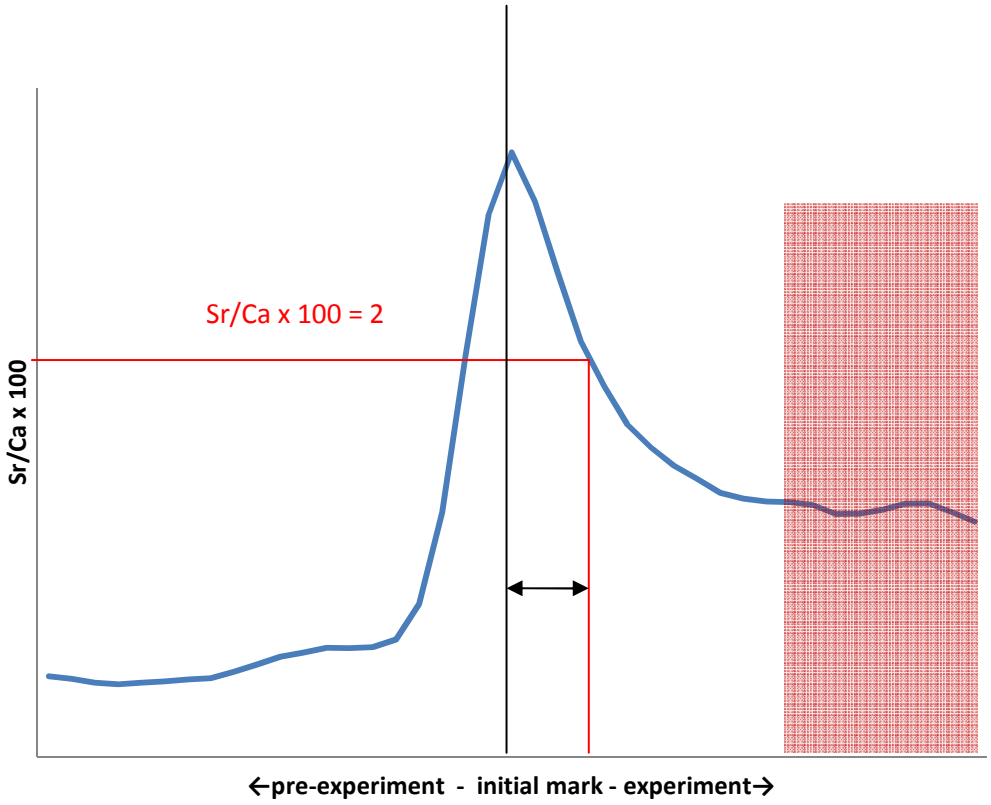

Fig. 7 Profile of Sr/Ca ratio from inside to the edge. The peak of the curve reflexes the initial Sr-marking. The red area marks the array where the Sr/Ca values are taken from. Requirement for the array was a plane reflecting steady state, or at least the smallest slope.

The shape of the curves was also analyzed to gain information about the time of Sr incorporation and therefore otolith growths. The values after the initial marking were used to perform a logarithmic regression. This was done by using the RGP function in Microsoft Excel (=RGP(Y-Values; LN(X-Values); ;WAHR). With the calculated regression curve (R^2 >0.9) the x-values for y=2 were determined. The x-values are labeled as "relative distance to mark" (RDM) and reflect the distance the otolith grew to a certain level of Sr incorporation. As only around ¾ of each otolith was measured, depending on the position of the otolith, the advantage of the used method compared to measuring the total distance from mark to the edge was that effect of posterior and anterior positioning of the otolith was decreased. To further evaluate otolith growths the presents or absents of the Sr/Ca-relevant area was tested for warm and cold treatments.

Raw Data Analysis of Field Sample Otoliths

The raw counts were interpolated and transferred into ppm and set as ratios like for the experimental otoliths. The measurement points were pooled together and labeled to a value by optical determination shown in Fig. 8. As the otoliths are chemically inert, the pre-experimental part of the otoliths from animals was considered to represent in situ conditions and Sr/Ca values of the field samples were assumed to correspond to the average salinities of the capture sites. Doverodde (S=25, (Ryves et al. 2006)), Fiskebäckskil (S= 24, pers. comm. O. Roth), Gelting Bay (S=17.123) and Kiel Fjord (S= 17.297). Average salinities of Kiel Bay locations were calculated over a timeframe of ten years (pers. communication HH Hinrichsen).

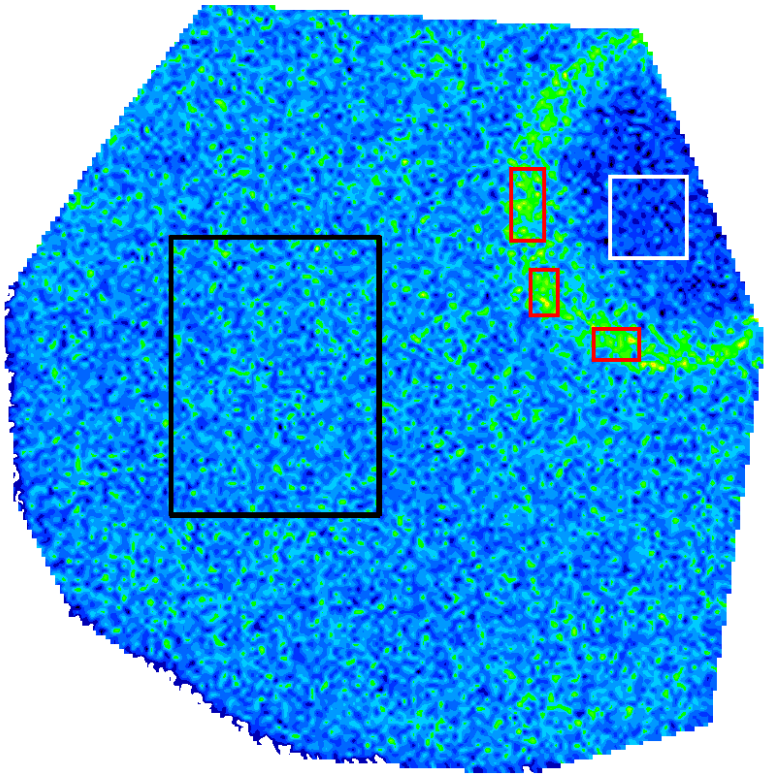

Fig. 8 Sample regions on the otolith. The black box provides the average Sr/Ca-ratio. The white box includes the values for the core data. The values in the red boxes are labeled as birthmark.

Further transects from the core to edge of otoliths of different life stages (juveniles 2-3 month, animals captured in spring) were analyzed for recognizable patterns, which could be related to migratory behavior during wintertime.

Statistics of Experimental Data

All statistics were performed with R (v. 2.13.2).

The values of the Sr/Ca-ratio for low salinity treatments were checked for normal distribution using the Shapiro-Wilk-Test (Shapiro and Wilk 2007). To check for a temperature effect an ANOVA was performed using the aov-function in R (Chambers and Hastie 1992). Homogeneity of variances was tested by the Fligner-Killeen-Test (Chambers and Hastie 1992; Wilkinson and C. E. Rogers 1973).

The correlation of Sr/Ca and salinity was checked for only the warm treatments. The Sr/Ca-values were tested for normally distribution with the Shapiro-Wilk-Test. The correlation was calculated with the linear regression function (lm) in R (Chambers and Hastie 1992; Wilkinson and C. E. Rogers 1973). Homogeneity of variances was tested by the Fligner-Killeen-Test.

Normally distribution of Sr/Ca-ratios and growth was tested by the Shapiro-Wilk-Test. Correlations have been checked by multiple regression. Homogeneity of variances was tested by the Fligner-Killeen-Test.

The values of the marking-profiles were analyzed for their dependence of salinity and temperature. The relative distance to mark data were checked with the Shapiro-Wilk-Test for normal distribution. Afterwards a multiple ANOVA were performed using the aov function of R. Homogeneity was tested by the Fligner-Killeen-Test. Relative distance to mark values have been tested the same way for temperature treatments.

Effect of somatic growth on relative distance to mark values was checked by a linear regression. The growth data had to be transformed using the tenth logarithm (log(growth+1)) to achieve normal distribution according to the Shapiro-Wilk-Test. In addition, the correlation between relative distance to mark and the Sr/Ca-ratio was tested. Normality was checked with the Shapiro-Wilk-Test and correlation tested using linear regression. Homogeneity of variances was checked with the Fligner-Killeen-Test.

Somatic growth was checked for correlation on temperature and salinity. Growth was checked for normal distribution using the Shapiro-Wilk-Test. Somatic growth was not normally distributed and therefore the data were transformed using the tenth logarithm (log(growth+1)). To check for correlations a multiple regression was performed using the lm function of R.

Otolith analyzability, thus how many of the dissected otoliths exhibit the required pattern for Sr/Ca-ratio analysis, was tested using the χ^2-test. Therefore cold against warm analyzability was tested.

The relationship between fish size and Sr/Ca-ratio was investigated. For doing so the otoliths of experimental fish were used, as for all of them the total length of the fish was

available. The data were transformed (log(length+1)) to reach normal distribution. To check for correlations of length to Sr/Ca-ratios a single regression was performed.

Statistics of Field Data

The dependence of Sr/Ca-ratios on salinity was studied including all otoliths. Normality was tested with the Shapiro-Wilk-Test. Within the animals of each sample station there is Gaussian distribution except for German animals. Two outliers have been detected using Dixon-Test for outliers (Dixon 1950, 1951). After removal of these values the data were normal distributed. The Fligner-Killeen-Test showed heterogeneity of variances. To achieve homogeneity the data were transformed on logarithmic scale (log(values+0.1)). Homogeneity of variances within the birthmark data could not be achieved neither by transformation. Therefore two single regressions for the average and the core Sr/Ca-ratio were performed to detect their dependence on salinity. Relationships between each of the measurement areas were analyzed by separate single regressions. Homogeneity of variances was tested by the Fligner-Killeen-Test.

All statistical tables can be found in the appendix.

Results

Experimental Part

During the experiment a higher activity for fish of the warm treatment could be observed. Indicating for higher activity also serves the number of animals accidentally jumped over the aquarium walls (12°C treatment N=7, 20°C treatment N=28), therefore death rate was much higher in the warm treatment (cold=30%, warm=50%). The cooling device of the 12°C/15 treatment broke down for two weeks, and the temperature could not longer be provided and the temperature raised to 18°C. During that time 30% of the animals accidentally jumped over the aquarium walls, compared to 11.3 % during 12°C. Despite the activity an individual acceptance of the defrosted mysidacea as food could be observed. The animals grew 1.08 cm ±0.59 in the cold treatment, and 1.12 cm ±0.67 in the warm treatment within ten weeks. Multiple regression concerning growth depending on salinity and temperature did not show a significant effect (for temperature p=0.71 and for salinity p=0.68).

	12°C / S 30	12°C / S 23	14°C / S 15	20°C / S 30	20°C / S 23	20°C / S 15
Otolith dissected + RDM	13	14	13	13	10	15
Otolith Sr/Ca	2	2	9	12	8	13
Growth	9	7	8	9	5	11
Growth+Sr/Ca	0	1	6	8	4	7

Tab. 2 Table shows how many otoliths were available for each analysis. For each dissected otolith relative distance to mark could be taken. Sr/Ca-ratio and somatic growth were not available for each otolith and both values were available for even less.

Sr/Ca-Ratio

For the 20°C treatment a positive correlation of the Sr/Ca-ratio and salinity was detected (single regression, R^2=0.1314; f=4.387; DF=1,29; p=0.045)(Fig. 9). Due to the limited growth of otoliths in the cold treatments, the data for those treatments were excluded from the analysis (Tab. 2). To further check for temperature effects the low salinity treatments were compared in an ANOVA, as for both treatments enough values were available. The ANOVA did not show a significant effect of temperature (ANOVA, DF=1,20; f=0.2779; p=0.604). Therefore temperature was as factor excluded for further Sr/Ca-ratio analysis.

Further, somatic growth was included in the analysis of experimental data. The dataset became smaller as growth data were not available for all animals. A significant negative correlation was found for growth, an effect for salinity (although the slope was not significant p=0.53836) and the interaction of growth and salinity (multiple regression, R^2=0.518; f=7.523; DF=3,21; p=0.001; salinity, DF=1,21, f=7.5576; p=0.012; growth, DF=1,21; f=9.7971; p=0.005; interaction, DF=1,21; f=5.5068; p=0.02836)(Fig. 10).

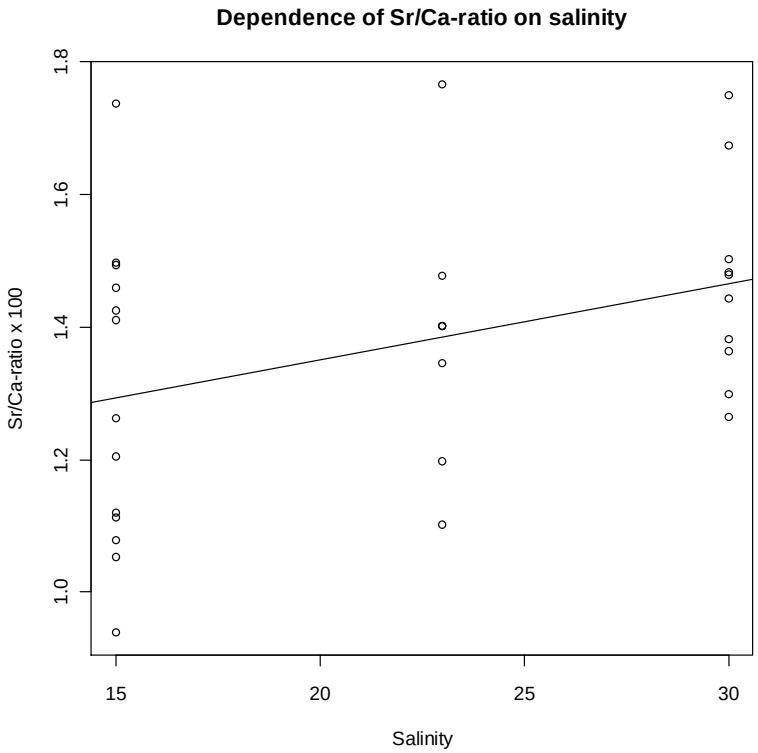

Fig. 9 Positiv correlation of Sr/Ca-ratio and salinity within the experiment. The slope of 0.011503 is significant

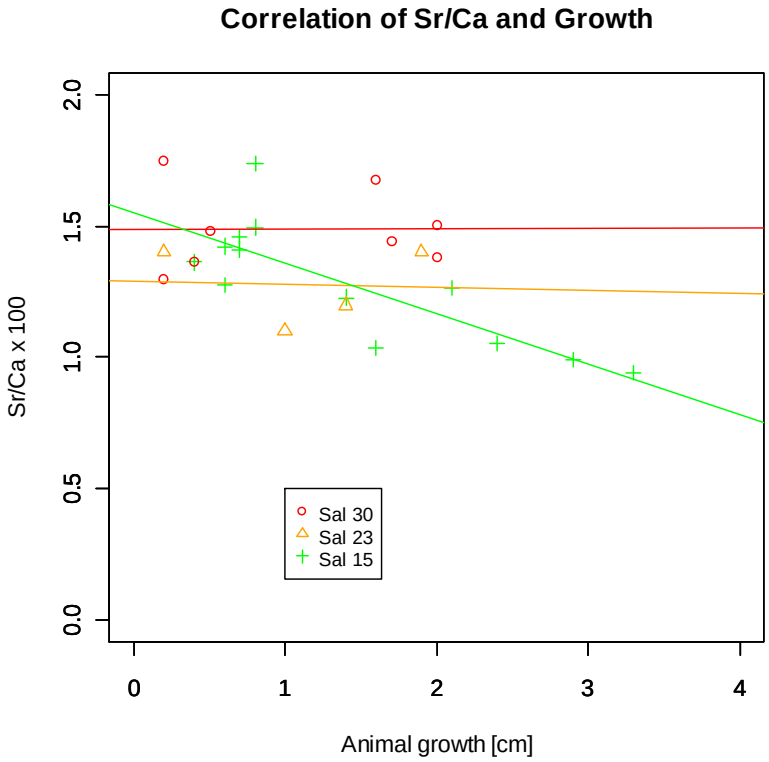

Fig. 10 Correlation of Sr/Ca-ratio on growth at different salinities. Only at salinity 15 there is a negative effect of somatic growths on Sr/Ca-ratio.

Marking Profiles

For the marking profile analysis all dissected otoliths could be used, including otoliths of the 12°C/30 and 12°C/23 treatments. A Multiple Analysis of variances of the marking-profiles gave a significant signal for temperature (MANOVA, DF=1,73; f=340.971; $p=1.349e^{-07}$) and the interaction term with salinity (MANOVA, DF=1,73; f=49.662; p=0.029)(Fig. 11). Salinity itself could not be detected for having a significant influence (MANOVA, DF=1,73; f=0.9003; p=0.34). For only the low salinity treatments an ANOVA gave a significant difference for temperature too (ANOVA, DF=1,24; f=47.467; p=0.039). With animal growth as continuous factor the chosen model was the simple regression, detecting no influence of animal growth (simple regression, $R^2=6.042e^{-06}$; $f=2.356e^{-04}$; DF=1,39; p=0.74). Also a significant correlation between Sr/Ca and relative distance to mark could be observed (single regression, $R^2=0.1287$; f=6.054; DF=1,41; p=0.018).

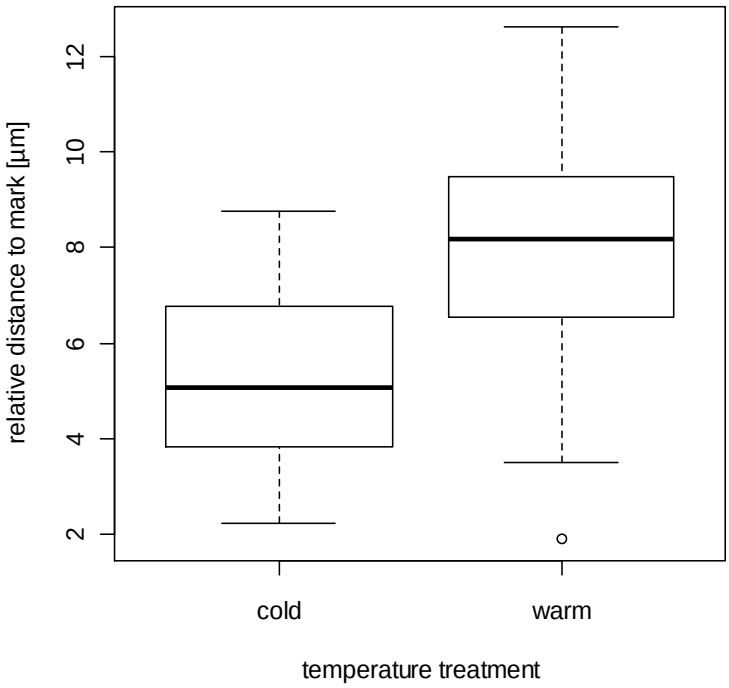

Fig. 11 Relative distance to mark on temperature. ANOVA is significant (p=1.349e-07) for temperature differences. Between cold (12°C and 14°C, N=40) and warm (20°C, N=38) treatments.

Only four otoliths of animals from treatment 12°C/30 and 12°C/23 could provide data. Growth of otoliths was deficient for analysis of the others. The average temperature of the cold treatment with a salinity of 15 rose due to problems with the cooling system to 14°C with a maximal temperature of 19 °C. From this treatment nine otoliths showed an analyzable pattern. From the warm treatment in significant more dissected otolith the required growth pattern was visible (X^2=23.785, df=1, p=1.077e^{-06}) (Tab. 2, Fig. 12).

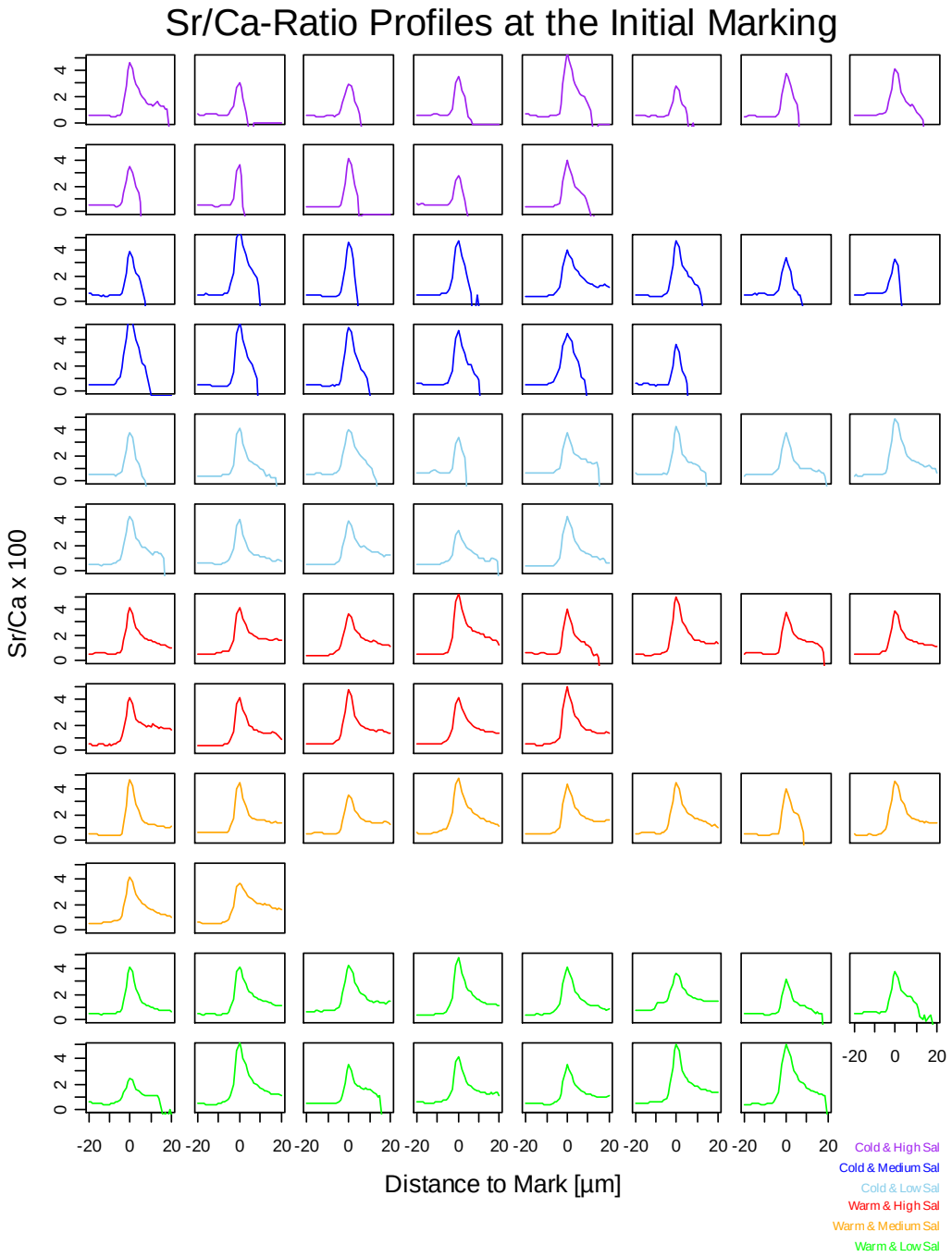

Fig. 12 Profiles of every measured otolith. Where the curve becomes null, the otolith edge was reached. Negative values on the x-axis are before (salinity ~17), positive after Sr-marking. The growth pattern described in Fig. 7 are only visible in treatment 12°C/15 and the 20°C treatments. The curves are still declining at the end.

Further otoliths of juveniles born during the experiment show a more intense birthmark than animals born in the Baltic (Fig. 13). Reason for this could be the intrinsic exposure to high Sr during as embryo in the broodpouch during the marking of the adults. Since there are only two otoliths available no statistical analysis was possible. Both otoliths show a different pattern of Sr/Ca-ratio decline. In one otolith the decline seems to reach a platform above before going down to the final level, while the other seem to decline directly to a final level.

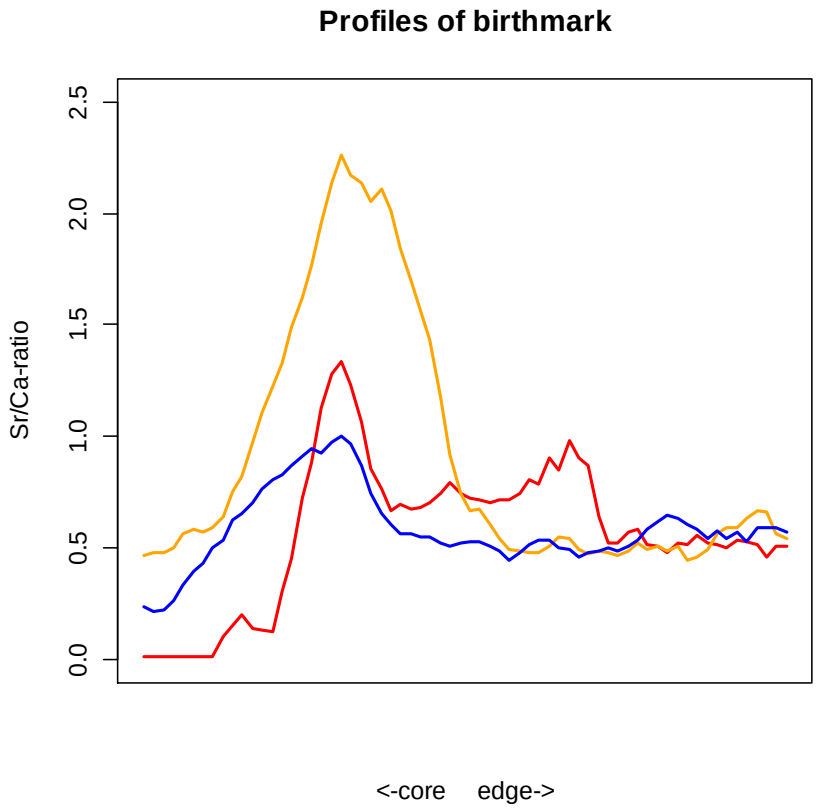

Fig. 13 Profiles of 2-3 month old juveniles born during the experiment (red and orange). The animals experienced a Sr-bath during embryonic development, therefore have a modified birthmark. In comparison a natural birthmark (blue).

Field Sample Otoliths

In field samples a relationship between water salinity and otolith Sr/Ca ratios were detected as well. The linear regression for the average salinity of the sampling site was highly significant correlated to average Sr/Ca (single regression, R^2=0.6794, f=135.3, DF=128, p<$2e^{-16}$) (Fig. 14) and to the core Sr/Ca (single regression, R^2=0.1838, f=24.22, RF=128, p=3.61^{-07}).

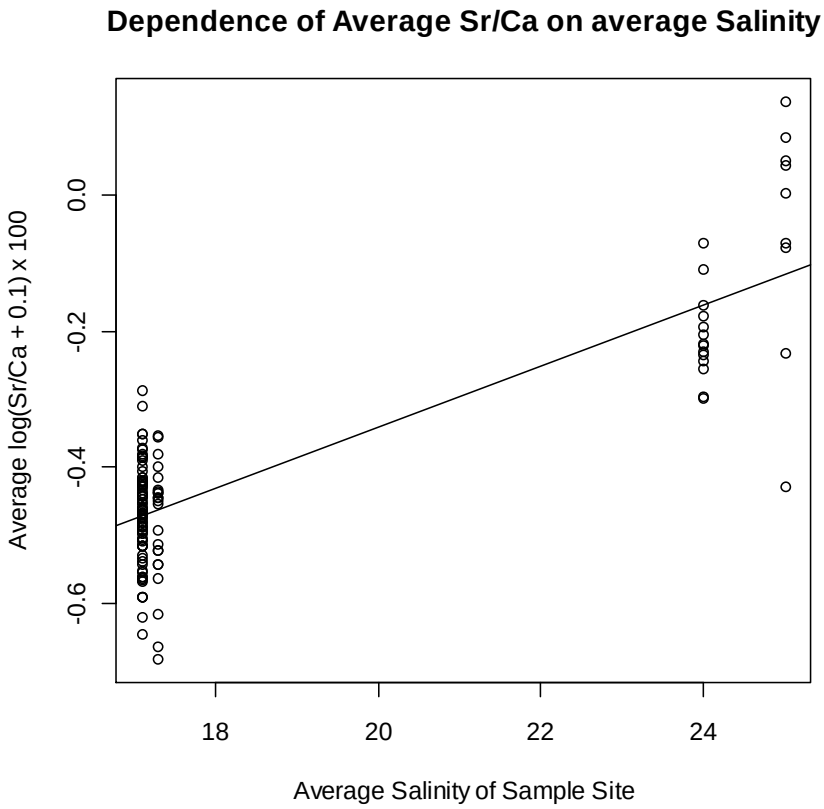

Fig. 14 Dependence of logarithmic Sr/Ca-ratio of natural otoliths on average salinity of the sampling site. The slope of 0.044746 is significant

Over all sampling sites all measurement sites show a positive correlation to each other (avg Sr/Ca:core, single regression, R^2=0.1582; f=24.06; DF=1,128; p=2.78e^{-06}; core:birthmark, single regression, R^2=0.4924; f=124.1; DF=1,128; p=1.42e^{-07}; birthmark:avg Sr/Ca, single regression, R^2=0.4924; f=124.2; DF=1,128; p<2e^{-016}) (Fig. 15, Fig. 16).

Regression analysis concerning length and average Sr/Ca-ratio could not detect a correlation (single regression, R^2= 0.0001017; f= 0.007528; DF=1,74; p= 0.9311).

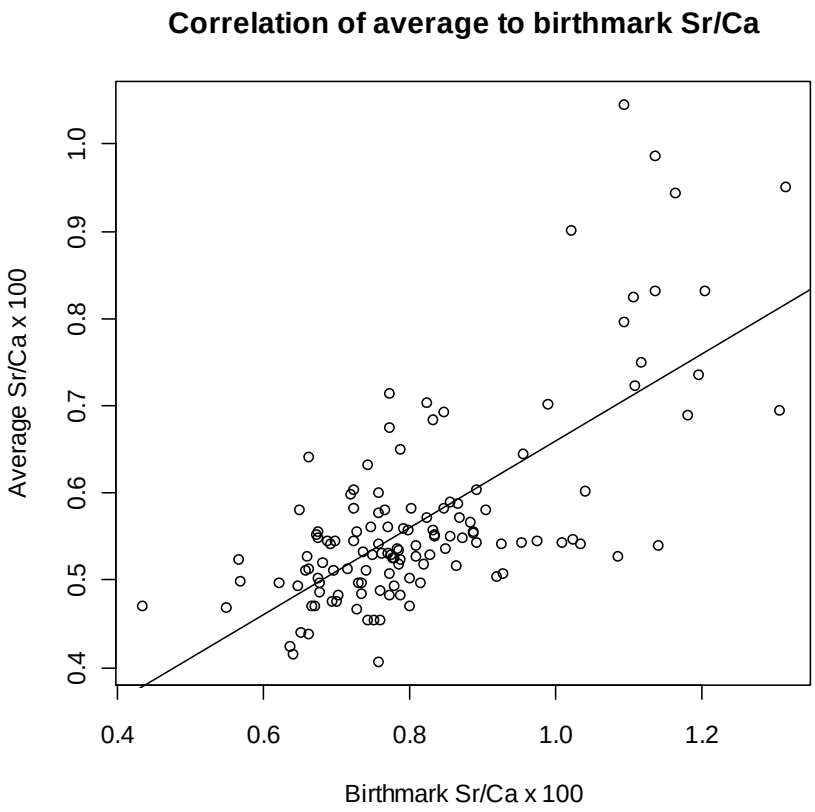

Fig. 15 Positive correlation between average Sr/Ca values and birthmark.

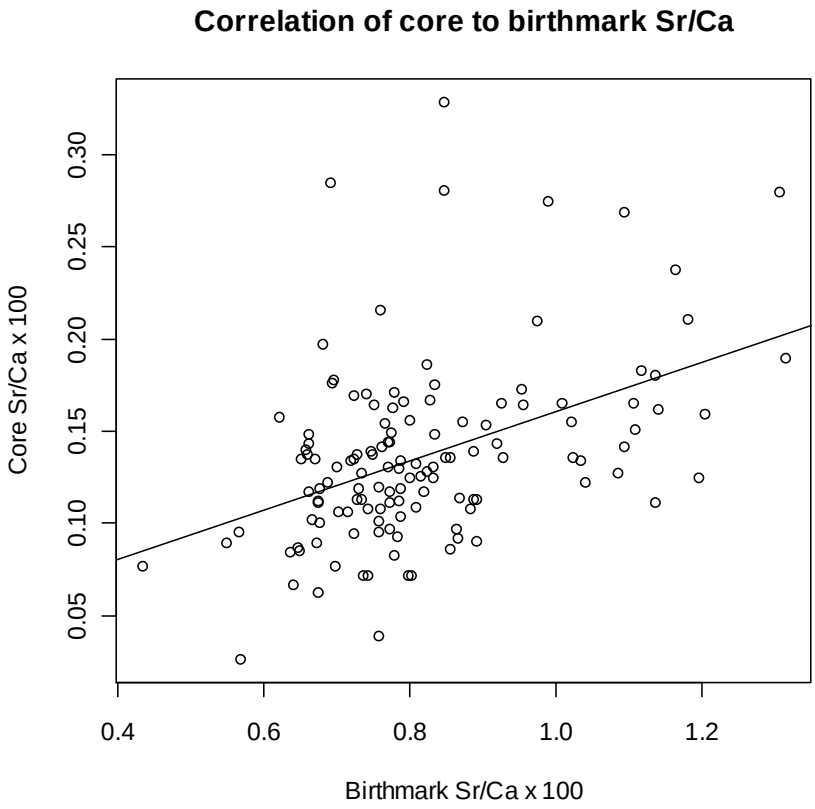

Fig. 16 Correlation of core to birthmark of animals from Denkmark, Sweden and Germany

Finally the profiles of natural otoliths from core to the edge were analyzed for natural Sr/Ca-markings, which refer to migratory behavior. However, over all otoliths only one Sr/Ca-marking was consisted, which was the birthmark. Marks related to migration during wintertime were not found (Fig. 17).

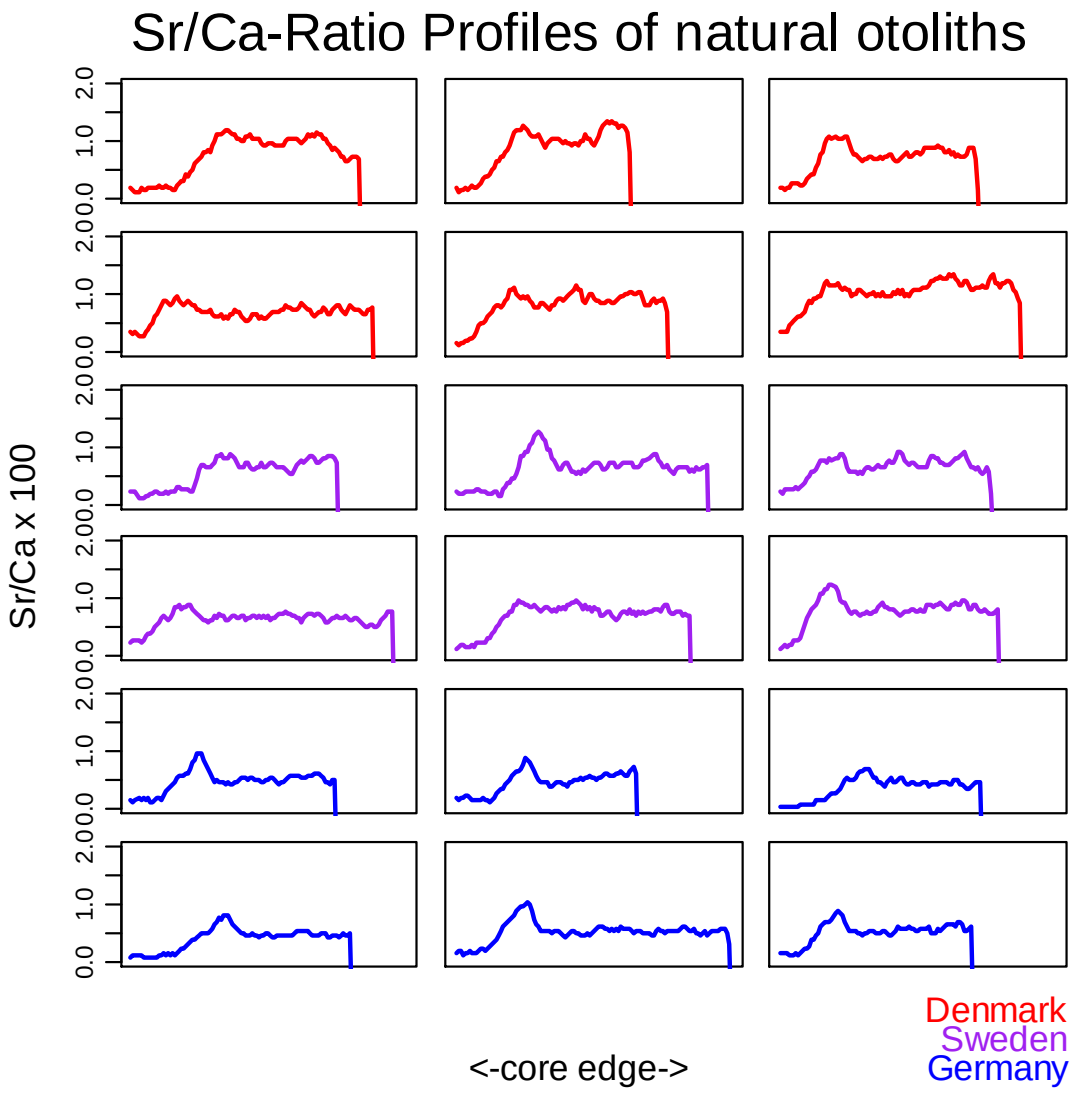

Fig. 17 Random extraction of profiles from natural otoliths. Profiles starts left with the core region. The following peak is the birthmark. No further consisted mark was visible.

Discussion

Influences on Sr/Ca-ratio Revealed by Experimental Data

The experiment allowed disentangling the relationships influencing the Sr/Ca-ratio, as the Sr/Ca-ratio is dependent on several influences. Salinity and animal growths have a direct effect on the Sr/Ca-ratio, while the temperature effect seems to be more an indirect effect which has already been suggested (Sadovy and Severin 1994). Over all the experiment showed that otolith microchemistry can detect environmental influences and hence is an appropriate tool to reconstruct migration associated patterns in otoliths of Syngnathids.

Bringing all experimental results together one ends up with following scheme:

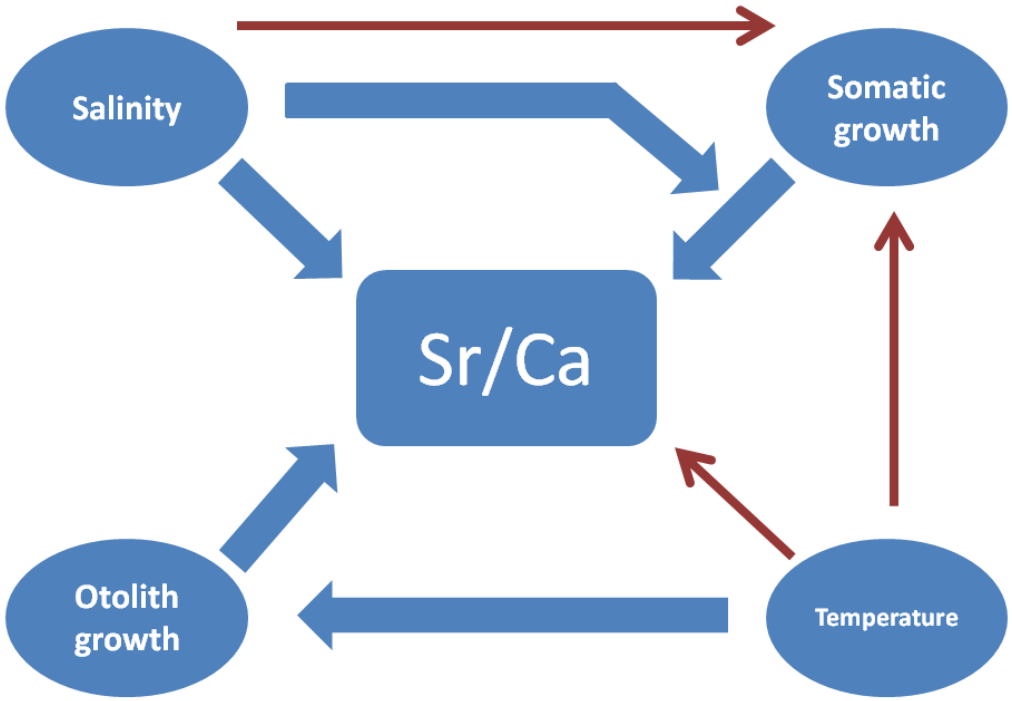

Fig. 18 Scheme of relationships influencing Strontium incorporation into otoliths. Blue arrows show correlations actually shown in the experiment while red arrows are only found in literature.

Direct Influences on Sr/Ca-ratio in Details

Salinity has been described as one of the major influences on Sr/Ca-ratio (Campana 1999; Thresher 1999), which confirm present results. However, the positive correlation between salinity and Sr/Ca has never shown before for Syngnathids, only for different species (Campana 1999; Fowler et al. 1995; Macdonald and Crook, 2010; Thresher 1999), and had to be shown for Syngnathids before interpreting natural marks in otoliths.

The correlation of otolith growth and the Sr/Ca-ratio has never shown before for Syngnathids, although it has been suggested for other species (Fowler et al. 1995). Problematic might be that otolith growth was measured as function of Sr incorporation to Ca accumulation, e.g. measured at the same Sr/Ca-ratio profile as the Sr/Ca-ratio itself. Therefore the correlation might be artificial. However, if this is the case then a direct correlation of temperature and Sr/Ca-ratio within the low salinity treatments would be predictable, as there is the correlation between average distance to mark and temperature. Since this is not the case and average distance to mark and Sr/Ca-ratio are dependent on different influences, it is assumable that the correlation is not artificial.

Even no effect of temperature could be shown at a salinity of 15 which does not necessarily exclude a temperature effect at higher salinity, as the effect of temperature has already been discussed as species specific and more important of interaction with salinity (Elsdon and Gillanders 2003).

Faster growing individuals tend to incorporate less Sr, but only at low salinity. This is quite surprising as the influence of growth had been described before, but for marine fish, so for high salinity (Sadovy and Severin 1994). Assumed that the growth effect is negatively correlated to salinity (larger effect on low salinity and vice versa), it is possible that in present experiment the growth effect in higher salinity treatments were just too small to detect.

Indirect Effects on Sr/Ca-ratio

However, in former studies clear effects of temperature and salinity on animal growth were suggested (Boeuf and Payan 2001; Imsland 2001; Killorn and Toews 2001; Mckay 1985), here no effect has been found. Possibly this emerged through the fact that the

main food source was not natural, hence, the acceptance for defrosted food might have caused the high scatter and therefore makes a significant differentiation of growth rate impossible.

In contrast, otolith growth is dependent on metabolic effects, like otolin production, but also on kinetic effects of calcium carbonate accumulation (Payan et al. 2004), which is demonstrated by the really strong effect of temperature. This implies that for ecological studies which take otolith growth into account to evaluate the nutritional condition (Clemmesen and Doan 1996), temperature has to be considered. For the same class this may not be that important, as they should all undergo the same temperatures, but for comparisons of different year classes or stocks this might be relevant.

Correlation between otolith growth and somatic growth has been described in a study about *Pristipomoides zanatus* (Ralston 1986). However, another study states that otolith growth and somatic growth are not synchronic (Labropoulou and Papaconstantinou 2000), what corresponds present findings. Otolith growth and somatic growth are both often but not necessarily correlated and further influenced by temperature. Hence the influence has to be stronger for one of them and since only dependence for otolith growth was shown the influence of temperature has to be stronger on it.

Comparison of Sr/Ca-values to natural average Sr/Ca

Beside the correlations, it should be mentioned that compared to Sr/Ca ratio measured in the otoliths from wild-caught animals that were not exposed to a Sr bath, the values from the experiment are increased, which is visible when comparing the pre mark curve with the after the mark. Further the highest values for natural otoliths were around 1.0 Sr/Ca x 100 and for the experiment 1.8.

In the experiment only salinity was measured and not Sr-concentration, which could be increased compared to natural conditions. Further the water in the experiment derived from the North Sea and the Sr/Salinity-ratio could in principal differ to the Baltic. However, due to technical problems the exact Strontium concentration could not be measured.

As alternate explanation for the increased Sr/Ca-ratio serves an acclimatization phase for the Sr/Ca-ratio (30 to 40 days) found by Macdonald and Crook (2010). Even though the present experiment run for 70 Days, the possibility still exists that the profiles shown in Fig.3 did not reach the steady state, in particular, because on the profiles the curve is still decreasing at the end of the experiment. 30 to 40 days were time frames for natural salinities, but instead the present marking was conducted with a 15 times higher Sr concentration in the water compared to a natural salinity of 35, besides the fact, that Macdonald and Crooks experiment was about a different species. Hence, it is likely that during the experiment the steady state of regeneration after Sr bath was not reached and that this explains the high Sr/ Ca ratio found in the experimental animals.

Interpretation of Natural Formed Pattern

Before interpreting natural marks in otoliths the time scales have to be evaluated in the otolith. To do so, otoliths of two 2-3 month old juveniles were analyzed. Within two months the otoliths possess already all three possible structures: core, birthmark and average Sr/Ca. It is likely that the birthmark is accumulated directly or at least relatively close to the birth as it describes the transit between the core, which is supposed to be formed during embryonic development, and the outer region of the otolith. Thus it is rather unlikely that the first Sr-ring is related to migratory behavior during wintertime.

To answer the question of migration during wintertime transects from the core to the edge of animals captured during spring have been analyzed with special concern to the region between the birthmark and the edge, respectively the Sr-marking. Within this transect the animals experienced winter. Any clear changes of the Sr/Ca-ratio would predict an environmental shift. However, no clear pattern could be observed, what indicates that with the help of otolith microchemistry migratory behavior cannot be explained. The second Sr-ring observed by Miersch *et al.* (unpublished) could not be detected and the reason for the absence/ or presence of this ring but also the meaning of it remains unclear.

The whole environmental patterns in the otolith represent the region (e.g. Kiel Bight, Kattegat and Limfjord) of the sample site, as average Sr/Ca, the core and the birthmark

are correlated over all sample sites. Hence, long migrations seem to be rather unlikely and therefore the genetic exchange, mentioned at the beginning, remains unexplained.

Besides the possibility that pipefish of the species Syngnathus typhle do not show any migration patterns , the method may just not be able to detect the migration pattern due to insufficient sensibility. Already at a rather high temperature of 12°C treatment the otolith growth was almost zero. When in winter the water temperature falls down to 4°C or even less, the accumulation of calcium carbonate is likely to be so marginal that the winter period may hardly be detectable using otolith microchemistry although these patterns might be there.

The only clear mark in the otoliths was the birthmark, which can potentially be caused due to metabolic effects during larval development (Campana 1999; Toole et al. 1993). However, environmental conditions can potentially affect and even cause the birthmark. The increased values for the birthmark of the juveniles experienced a Sr-marking during embryonic development indicate that the environmental conditions during embryonic development affect the birthmark respectively the otolith, which already has been shown (Kuroki et al. 2010; Shippentower et al. 2011). The core Sr/Ca is correlated to the average salinity of the captivity site. This also indicated that environmental conditions already influence the elementary composition of the otolith during embryonic development. Possibly both have an influence, metabolic effects during development as well as the environmental conditions.

Conclusion

Otolith microchemistry is an appropriate tool to investigate environmental changes in Syngnathids, as the incorporation of Strontium into the otolith is dependent on salinity, otolith growths and at low salinities on somatic growth, while temperature has only an effect on otolith growth, and therefore an indirect effect on the Sr/Ca-ratio.

A clear answer to the question of migratory behavior is not possible with otolith microchemistry. Large scale migration as observed for *S. fuscus* in the Mid-Atlantic Bight with large distances with more than 100 km in higher saline deeper waters are unlikely,

unless otolith growth would be too small to detect these patterns (Lazzaril and Able 1990). For further investigations on the overwintering habitat of *S. typhle* one should concentrate on different approaches, maybe the possibility that the migration might be temperature controlled (Lazzaril and Able 1990). Therefore a hydrographic model would be useful to predict warm water areas during the wintertime. Verification of pipefish occurrence could be done by trawling.

Chapter 2: Migrations and niche use of Syngnathids assessed by stable isotope analysis

Beside the information for migration even more information about the ecological role of a fish is comprised in the analysis of stable isotopes. However, to get this information, one cannot concentrate on single species, but rather include other species depending on the question asked.

According to the niche theory species that coexist have to fill different ecological niches elsewise one species would outcompete the others (Bastolla et al. 2005; Hutchinson 1957). Ecological niches are not necessarily a matter of space, rather a description of the role of a species within an ecosystem. Ecological niches are important to explain speciation without geographical barriers (Turelli et al. 2001).

S. typhle, *S. rostellatus* and *N. lombriciformi* are three pipefish species which are usually found coexistent within the eelgrass meadows of northern Europe. Pursuant to niche theory these species have to fill a different ecological niche, which several studies suggest as gut content is related to snout form and species show ontogenetic spatial occurrence in the eelgrass bed (Franzoi et al. 2004; Malavasi et al. 2007; Oliveira et al. 2007; Vincent et al. 1995). So far niche theory has not been tested for Syngnathids and hence present study aims to fill this gap on the basis of stable isotope analysis.

Introduction into Stable Isotope Analysis

Gut content analysis is always a snap-shot, concerning the trophic level of an animal (Feuchtmayr and Grey 2003). Some animals digest their prey quickly, and therefore make identification difficult, beside the fact that such prey then might be underestimated and the gut may contain material which is not assimilated (Lajtha and Michener 1994). Stable isotope analysis avoids these problems as it integrates over time and therefore perfectly expands the possibilities to detect trophical interactions, although it also covers not all possible questions on food web ecology. The taxonomic resolution of stable isotope analysis is rather low and also short term food changes

cannot be detected with this method. Hence gut content and stable isotope analysis complement one another (Feuchtmayr and Grey 2003; Renones et al. 2002).

Each element consists not only of one type of isotope, but is rather a mixture of different atoms which differ in the number of neutrons but have the same number of protons. Some isotopes are radioactive and decay with the time (e.g. ^{14}C), which provides a tool for age determination, while other isotopes are stable (^{13}C / ^{12}C or ^{15}N / ^{14}N).

How can this be used as tool for trophic level identification? The reason is fractionation. The ratio between stable isotopes is not fixed and is changed with every physical change in state of aggregate and every chemical reaction. For equilibrations the general rule is that heavier isotopes concentrate in the molecule where the bond strength are greatest if the conditions are of free exchange (Peterson and Fry 1987). Biological processes are more complex than simple equilibrations and involve kinetic isotope effects, where heavy isotopes are discriminated against light isotopes, because light isotopes react faster (Peterson and Fry 1987).

In food web analysis and trophic classification the fractionation of each enzymatic reaction is less important than the overall fractionation of isotopes from prey to predator. The ratio of stable isotopes in nitrogen ($\delta^{15}N$) is typically 3-4‰ higher in a consumer relative to its diet and therefore suitable to identify the trophic level of the consumer (Peterson and Fry 1987; Post 2002), while in contrast for carbon ($\delta^{13}C$) there is little change between consumer and diet and therefore often used to identify the base of a food web, for instant study benthic or pelagic primary producers (Post 2002).

The time scale which is represented in the measurement is dependent on the tissue used for analysis. Stable Isotopes of calcified body parts represent often a long time scale, as tissue turnover is slow (Tieszen et al. 1983). Otoliths and scales are even chemically inert and therefore represent the time of formation (Campana 1999). Muscle tissue covers a time frame of about weeks to months, as this tissue is renewed relatively slowly, with the duration generally positively correlated with body size of an organism (Tieszen et al. 1983).

Concerning migratory behavior stable isotopes of muscle tissue can reflect a habitat shift, if different food sources are used. Often this is represented in a shift in $\delta^{13}C$, as of different food webs the basic food source is different (Hobson 1999). Pelagic food webs for example tend to be more depleted in ^{13}C compared to inshore food webs (Hobson 1999).

In this study stable isotope analysis was used to verify niche formation and investigate possible food shift during wintertime.

Material and Methods

Animals were collected over in fall 2009, spring 2010 and in 2011 in spring and fall on different sites. While Falckenstein (Germany; +54° 24' 15.55", +10° 11' 35.68") and Strande (Germany; +54° 26' 4.23", +10° 10' 12.10") are close in the Kiel Fjord, Eckernförder Bay (Germany; +54° 33' 14.75", +10° 1' 49.68") is 20-30 km away (Fig. 19). The animals were killed by cutting the head of. Fish were measured for total length.

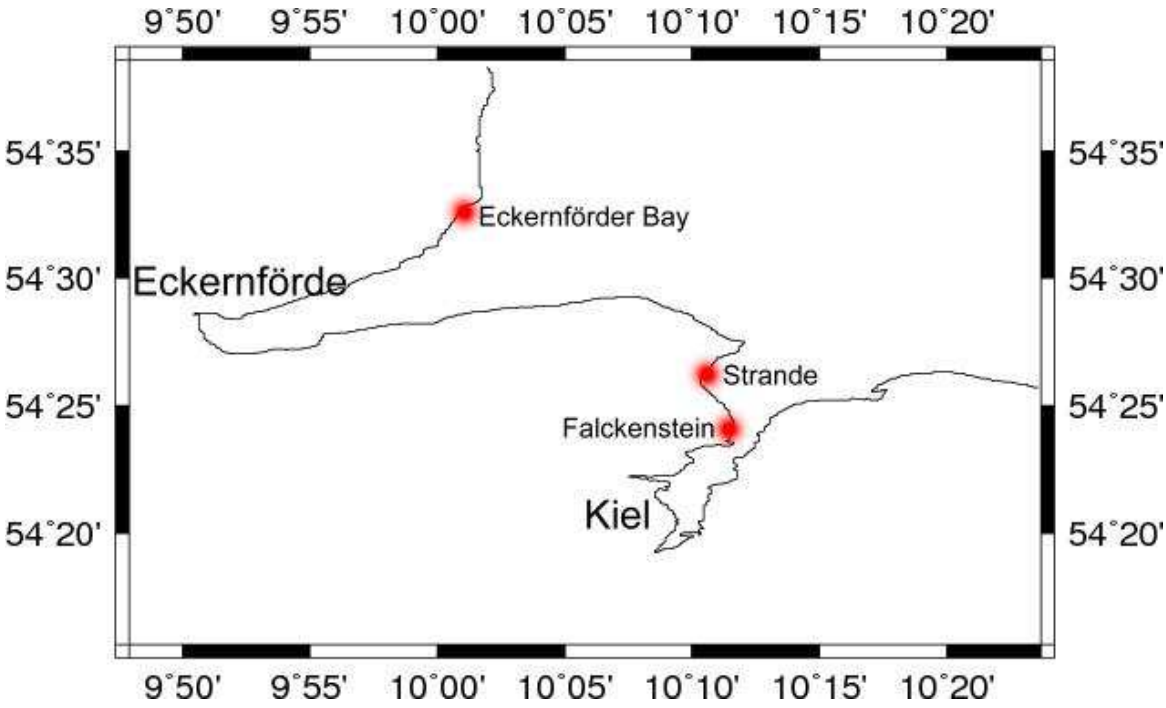

Fig. 19 Sampling sites for animals used for stable isotope analysis. (Graphic from: NOAA Coastline Extractor)

Sample Preparation

The muscles were dissected under the stereo microscope. Therefore the fish were opened at the ventral site and the intestines were removed. Afterwards the vertebral column was picked out by cutting on the sides of it and taken it out with forceps. The muscles were scratched from the proximal site of the bony skin with a scalpel. The extracted muscle tissue was freeze dried for 24 hours and further pulverized and approximately one milligram tissue filled in tin cups (HEKA tech.) and weighted. The tin cups with the tissue were folded to small pellets and put into a 96-well plate which was covered with parafilm to protect the pellets from falling out again. The filled well plate was send to Davis Stable Isotope Facility for C/N analysis.

The samples were analyzed with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Combustion of the samples took place in a reactor packed with chromium oxide and silvered cobaltous/cobaltic oxide at 1000°C. In the next step in a reduction reactor (reduced copper at 650°C) oxides were removed. The helium carrier then flowed through a water trap (magnesium perchlorate). N2 and CO2 were separated on a Carbosieve GC column (65°C, 65 mL/min) before entering the IRMS. The standard materials were Vienna PeeDee Belemnite (vPDB) for δ^{13}C and atmospheric nitrogen for δ^{15}N.

Statistics

Normal distribution for continuous factors was tested by the Shapiro-Wilk-Test. Length data was transformed (log(growth+1)) in order to reach normal distribution. δ^{13}C was chosen as response variable for an ANCOVA with capture site, season and species as categorical factors and transformed length as continuous factor. The model was simplified using the step-function in R to reduce irrelevant interaction terms (Chambers and Hastie 1992). The homogeneity of variances was tested with the Fligner-Killeen-Test. For δ^{15}N the same statistical model was calculated. The simplified models were checked by ANOVA and AIC for their explanatory relevance (Sakamoto et al. 1986).

Results

The model for $\delta^{13}C$ (R^2=0.6685; f=6.483; DF=14,45; p=6.995e^{-07}) shows significant influences of season (DF=1,45; f=35.8885; p=3.22e^{-07}), species (DF=2,45; f=17.4568; p=2.45e^{-06}) and the interaction of species and length (DF=2,45; f=4.5655; p=0.015)(Fig. 20, Fig. 21).

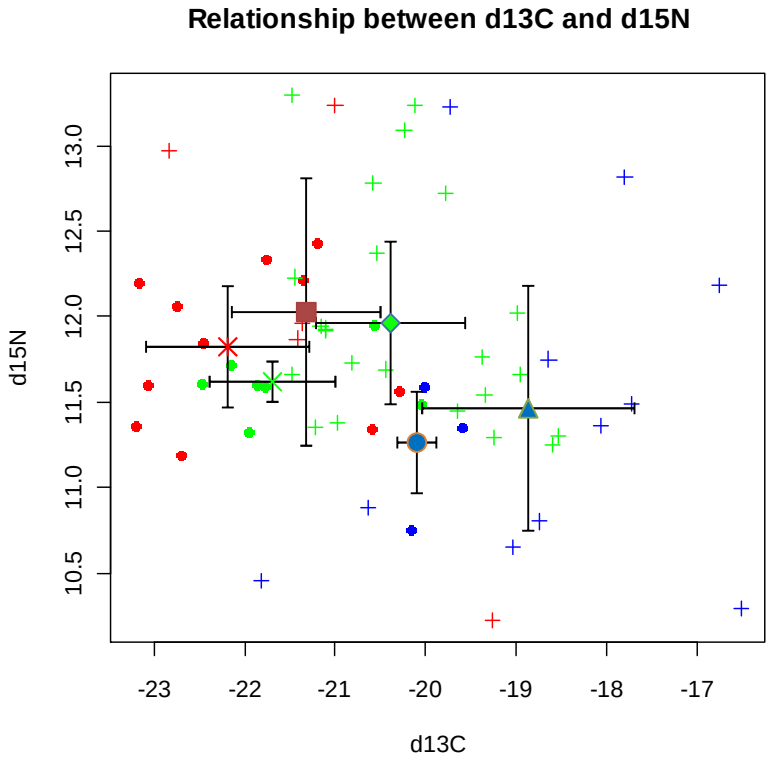

Fig. 20 C to N plot separated on species (green = *Syngnathus typhle*; red = *Syngnathus rostellatus*; blue = *Nerophis lombriciformi*) and season (+ = fall, • = spring). Big symbols are the averages with SD (spring = X X ● ; fall = ■ ◆▲)

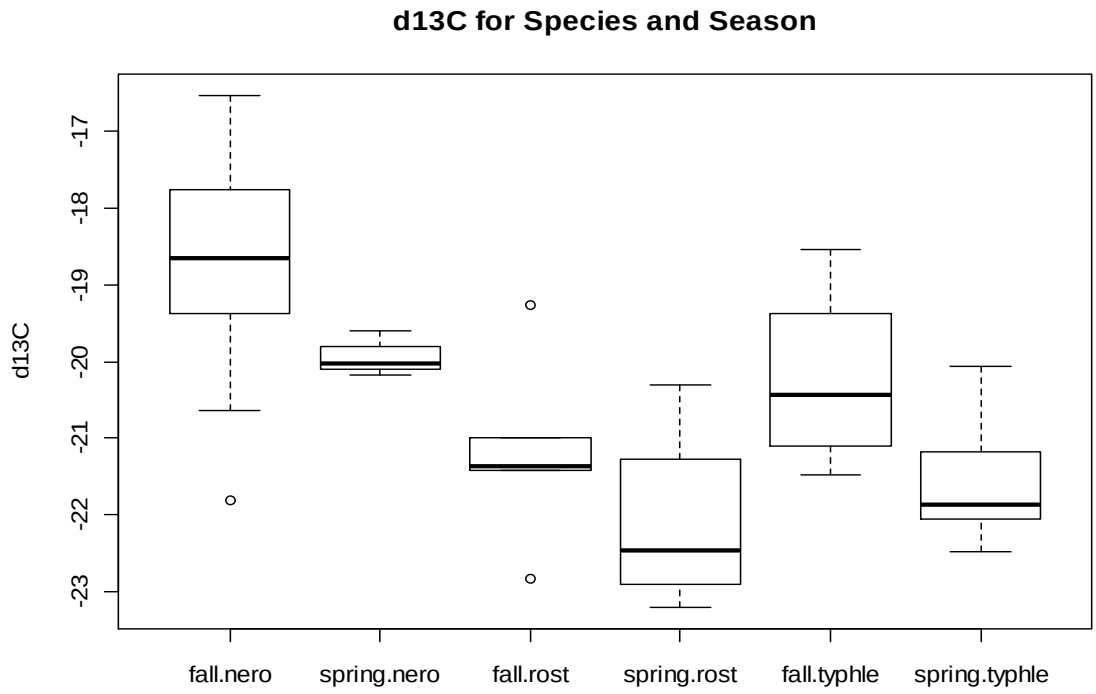

Fig. 21 $\delta^{13}C$ for species and season for animals captured in Kiel Bay.

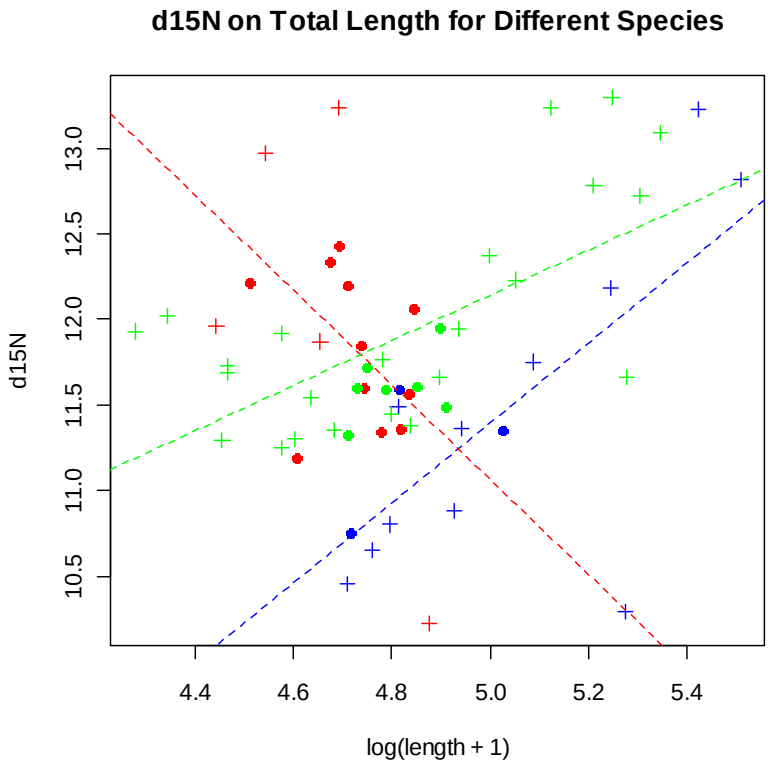

Fig. 22 N on total length of animals captured in Kiel Bay. (green = *Syngnathus typhle*; red = *Syngnathus rostellatus*; blue = *Nerophis lombriciformi*)

For $\delta^{15}N$ the model (R^2=5.226; f=6.081; DF=9,50; p=9.96e^{-06}) the influence of species (DF=2,50; f=4.605; p=0.015), length (DF=1,50; f=21.7; p=2.39e^{-05}) and the interaction (DF=2,50; f=7.349; p=0.002) are significant (Fig. 22).

Discussion

All three species of Syngnathids analyzed in this study showed significant differences in $\delta^{13}C$ and $\delta^{15}N$. These findings indicate a strong niche formation between these three species, which has not been shown before and therefore supports predictions from niche theory. This underlines the findings of Franzoi et al. 2004 who postulate that their findings of different gut contents are related to the snout form, while present results consider the trophic position in the food web. However, these niches are also spatial, as each species has its preferences in depth, height and density of eelgrass leaves (Malavasi et al. 2007; Vincent et al. 1995), what favors ongoing coexistence rather than outcompeting each other.

Ontogenetic shifts are common in the development of fish, as with growth larger prey items become available and therefore the fish rises in trophic level. For *S. typhle* and *N. lombriciformi* an increase of $\delta^{15}N$ with animal length was found, which confirms that in general with larger body size the possible prey size also increases and therefore reach a higher trophic level with size (Franzoi et al. 2004; Oliveira et al. 2007). Based on gut analysis Oliveira et al. (2007) found a major food shift between 10 to 12 cm total length for animals in Portugal, while present results would predict a higher trophic level and therefore a food shift more at length of 14 to 16 cm total length (Fig. 23). An explanation for this difference would be the different environment, as gut content analysis of *S. typhle* in Kiel Fjord show a higher content of Decapods and Fish in the diet (Bobsien 2006), compared to the results from Portugal.

For *S. rostellatus* the picture is not that clear. In general *S. rostellatus* preys primarily on small crustaceans like isopods and amphipods, but also on gastropods larvae (Kellnreitner et al. 2011). The negative correlation for $\delta^{15}N$ and length found in *S. rostellatus* is not significant (p= 0.770), and therefore it can be assumed that all *S. rostellatus* in this study represent one size class. The high variance of $\delta^{15}N$ between

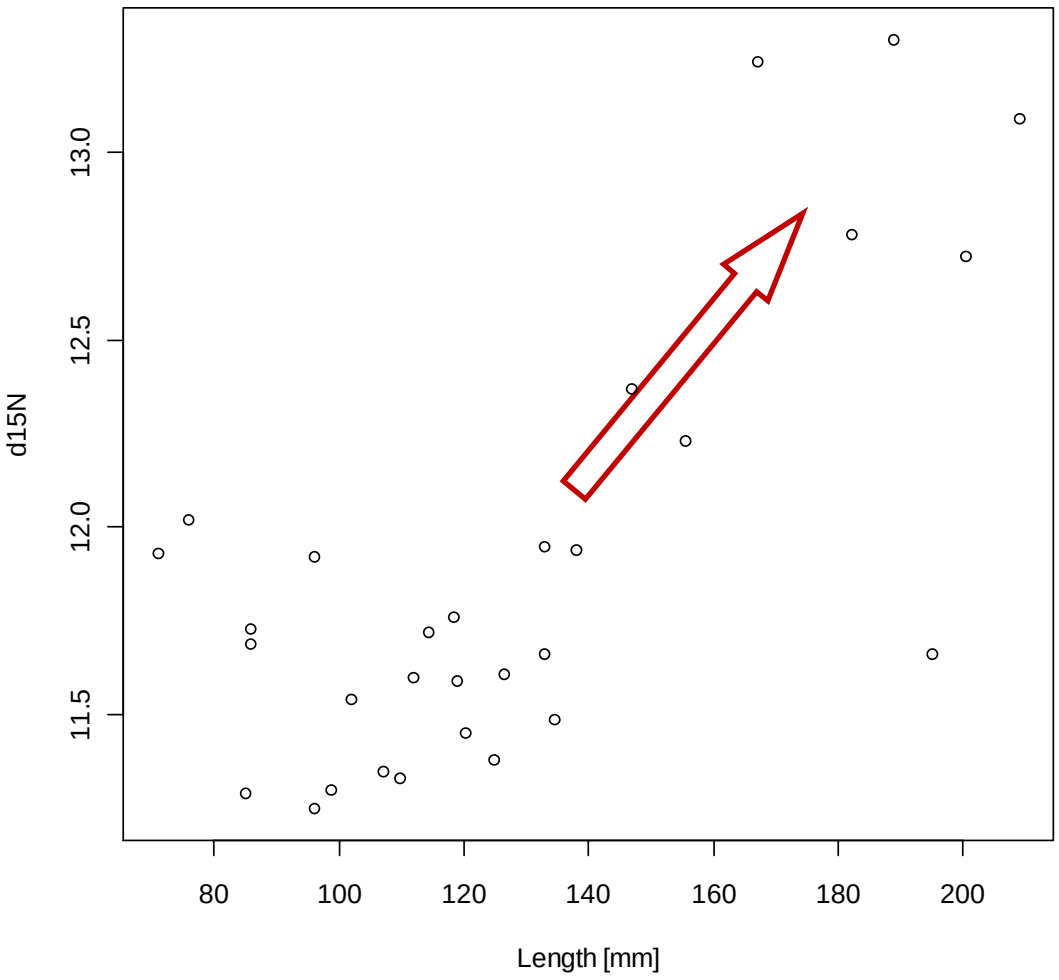

Fig. 23 Food shift according to d15N values.

animals of the same size class may indicate a more general feeding behavior of *S. rostellatus* compared to *S. typhle* and *N. lombriciformi*.

The relation between different basic food sources is different for each species, as the $\delta^{13}C$ is different. The relations of basic carbon sources for *S. typhle* have been analyzed by Jaschinski, Brepohl and Sommer (2011). They divide the basic food source in sand microflora, epiphytes and red algae, which might be problematic for *S.typhle*, as *S.typhle* is suggested to feed mainly on pelagic prey (Franzoi et al. 2004). According to own observations from animals held in aquaria, *S. typhle* favor pelagic over benthic food. For mysis, which is one of the food sources of *S. typhle*, suspended particular organic matter (pelagic source) makes ob to 40-70% of diet (Lesutiene et al. 2008). Assumed that $\delta^{13}C$

is rather conservative in the food chain, a comparison between benthic and pelagic average $\delta^{13}C$ values will provide and answer for the main food source, as eelgrass and epiphytes range from -15 to -10‰ $\delta^{13}C$ (Jaschinski et al. 2011)and pelagic plankton ranges from -20 to -18‰ $\delta^{13}C$ (Goering et al. 1990), which is much closer to the values found for *S. typhle*. Hence, for *S. typhle* a higher influence of pelagic plankton is assumable.

In *N. lombriciformi* the values for $\delta^{13}C$ are heavier compared to the genus *Syngnathus,* which then would predict a higher influence of a benthic carbon source, what fits the observations of Franzoi et al. (2004) and their conclusions on the snout form. In contrast, for *S. rostellatus* the lightest carbon ratio is found, which suggest *S. rostellatus* to be highly influenced by pelagic food sources.

A food shift which is necessarily associated to migratory behavior of *S. typhle* is not predictable, although the pelagic footprint in $\delta^{13}C$ favors a pelagic orientated residence during wintertime. To predict a food shift in wintertime one need the seasonal variability of the potential food sources. For pelagic plankton an increase in $\delta^{13}C$ from spring to fall has been observed (Goering et al. 1990; Rolff 2000), but also for eelgrass and epiphytes (Jaschinski et al. 2011), which is reflected in the isotopic carbon composition of Syngnathids. In spring the carbon composition is generally lighter compared to fall, which is ,in addition, unlikely related to starvation, as for starvation no effect or enrichment in heavy isotopes has been reported (Gaye-Siessegger et al. 2007; Gorokhova and Hansson 1999; McCue and Pollock 2008).

Final Conclusions

Within both approaches no definite pattern, which would allow determining the habitation of pipefish during wintertime, could be found, although some details to the life of *S. typhle* are assumable. Otolith microchemistry shows regional patterns, which indicates small distances for migratory behavior, while stable isotope analysis shows a principally pelagic pattern. Hence, a suitable scenario would be that pipefish stay in the pelagic relatively close to their ancient eelgrass bed. However this is not scientific verified.

In between Syngnathids a strong occupation of ecological niches was found, which is supported by gut content analysis (Bobsien 2006; Franzoi et al. 2004; Vizzini and Mazzola 2003, 2004), also that there is a change of the ecological niche during lifetime, what has already been described based on gut content analysis (Oliveira et al. 2007). This underlines the importance of Syngnathids for the ecosystem eelgrass meadow, as they fill several niches.

A legitimate question for pipefish and their time outside the eelgrass is about predatory pressure. Pipefish are perfectly adapted to the habitat eelgrass meadow, as they mimic eelgrass leaves (Vizzini and Mazzola 2004). Outside the eelgrass the advantage is gone and one would assume that there is high predatory pressure. Kleiber, Blight, Caldwell and Vincent (2010) suggest based on review of 135 papers where Syngnathids were recorded as prey that no predator is specialized on pipefish. A further conclusion was that pipefish are only eaten if nothing else is available because of their low caloric value. If this is truly the case, then predatory pressure would be quite low for Syngnathids outside the eelgrass beds, and one has to consider if the similarity of the body to eelgrass leaves is to hide from potential predators or maybe to hide from their own prey and therefore minimize feeding effort. As there was no evidence for starvation during wintertime, it is assumable that pipefish may be active feeders during wintertime. If pipefish can survive and feed outside the eelgrass, then the residence in the eelgrass might serve to reduce feeding effort and therefore to gain energy for reproduction. This is something further investigations may focus on.

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Appendix

Stat.Tab.1 Single Regression, Sr/Ca-ratio ~ Salinity

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Residuals:
      Min          1Q          Median 3Q          Max
      -0.35343     -0.16920     0.01402      0.12557      0.44457

Coefficients:
             Estimate     Std.Error    t value      Pr(>|t|)
(Intercept)  1.119.877    0.125389     8.931        8.03e-10     ***
salinity     0.011503     0.005492     2.094        0.0451       *

Residual standard error: 0.1969 on 29 degrees of freedom
Multiple R-squared: 0.1314 Adjusted R-squared: 0.1014
F-statistic: 4.387 on 1 and 29 DF, p-value: 0.04507
```

Stat.Tab.2 ANOVA, Sr/Ca-ratio (low Salinity treatment) ~ Temperature

```
             Df     Sum Sq       Mean Sq      F value      Pr(>F)
temperature  1      0.01468      0.014685     0.2779       0.6039
Residuals    20     105.673      0.052837
```

Stat.Tab.3 Multiple Regression, Sr/Ca-ratio ~ Salinity and Growth

```
Residuals:
      Min          1Q          Median       3Q          Max
      -0.30022     -0.08509     -0.00167     0.06451      0.36073

Coefficients:
                   Estimate     Std.Error    t value      Pr(>|t|)
(Intercept)        1.600.508    0.187035     8.557        2.75e-08     ***
salinity           -0.005233    0.008365     -0.626       0.53836
growth             -0.375788    0.119670     -3.140       0.00494      **
sal:growth         0.012907     0.005764     2.239        0.03610      *

Residual standard error: 0.1598 on 21 degrees of freedom
Multiple R-squared: 0.518, Adjusted R-squared: 0.4491
F-statistic: 7.523 on 3 and 21 DF, p-value: 0.00133

Analysis of Variance Table

Response: Sr/Ca-ratio
           Df  Sum Sq  Mean Sq F value  Pr(>F)
salinity    1 0.18760 0.187598  7.5576 0.01171 *
growth      1 0.24319 0.243189  9.7971 0.00487 **
sal:growth  1 0.13669 0.136692  5.5068 0.02836 *
Residuals  22 0.54609 0.024822
```

Stat.Tab.4 MANOVA, Relative Distance to Mark ~ Temperature and Salinity

```
             Df     Sum Sq       Mean Sq      F value      Pr(>F)
temperature  1      142.711      142.711      340.971      1,35E-04     ***
salinity     1      3.768        3.768        0.9003       0.34583
temp:sal     1      20.786       20.786       49.662       0.02893      *
Residuals    73     305.536      4.185
```

Stat.Tab.5 ANOVA, Relative Distance to Mark (Low Salinity Treatment) ~ Temperature

```
             Df     Sum Sq Mean Sq       F value      Pr(>F)
temperature  1      17.115 171.148       47.467       0.03941      *
Residuals    24     86.535 36.056
```

Stat.Tab.6 Single Regression, Relative Distance to Mark ~ Growth

```
Residuals:
      Min          1Q          Median       3Q          Max
      -3.937       -1.577       -0.120       1.498       4.136

Coefficients:
                  Estimate     Std.Error    t value      Pr(>|t|)
(Intercept)       668.826      0.67152      9.960        2.87e-12     ***
log(growth+1)     0.01324      0.86232      0.015        0.988

Residual standard error: 2.055 on 39 degrees of freedom
Multiple R-squared: 6.042e-06, Adjusted R-squared: -0.02563
F-statistic: 0.0002356 on 1 and 39 DF, p-value: 0.9878
```

Stat.Tab.7 Single Regression, Average Sr/Ca-ratio ~ Salinity

```
Residuals:
     Min           1Q           Median       3Q           Max
       -0.31074     -0.05704     0.00757      0.04968      0.25478

Coefficients:
             Estimate     Std. Error t value  Pr(>|t|)
(Intercept)  -1.237143    0.050619  -24.44    <2e-16       ***
sal          0.044746     0.002717   16.47    <2e-16       ***

Residual standard error: 0.08594 on 128 degrees of freedom
Multiple R-squared: 0.6794,     Adjusted R-squared: 0.6769
F-statistic: 271.3 on 1 and 128 DF,  p-value: < 2.2e-16
```

Stat.Tab.8 Single Regression Core ~ Salinity

```
Residuals:
      Min          1Q           Median       3Q           Max
      -0.57592     -0.10864     -0.00889     0.08994      0.65525

Coefficients:
             Estimate     Std. Error   t value      Pr(>|t|)
(Intercept)  -2.014238    0.104060     -19.357      < 2e-16      ***
sal          0.029982     0.005585     5.369        3.61e-07     ***

Residual standard error: 0.1767 on 128 degrees of freedom
Multiple R-squared: 0.1838,     Adjusted R-squared: 0.1774
F-statistic: 28.82 on 1 and 128 DF,  p-value: 3.606e-07
```

Stat.Tab.9 Single Regression, Average Sr/Ca ~ Core

```
Residuals:
      Min          1Q           Median 3Q           Max
      -0.18936     -0.05924     -0.02747     0.03031      0.37524

Coefficients:
                  Estimate     Std.Error    t value      Pr(>|t|)
(Intercept)       0.44278      0.02753      16.084       <2e-16       ***
core              0.93322      0.19025      4.905        2.78e-06     ***

Residual standard error: 0.1051 on 128 degrees of freedom
Multiple R-squared: 0.1582, Adjusted R-squared: 0.1517
F-statistic: 41084 on 1 and 128 DF, p-value: 2,78E-03
```

Stat.Tab.10 Single Regression, Birthmark ~ Average Sr/Ca

```
Residuals:
      Min       1Q       Median    3Q       Max
      -0.28673 -0.07797   -0.00353  0.05508  0.36577

Coefficients:       Estimate      Std. Error  t value      Pr(>|t|)
(Intercept)         0.25498       0.05158     4.943        2.36e-06     ***
avg Sr/Ca            0.98884      0.08874     11.143       <2e-16       ***

Residual standard error: 0.1151 on 128 degrees of freedom
Multiple R-squared: 0.4924, Adjusted R-squared: 0.4884
F-statistic: 124.2 on 1 and 128 DF,  p-value: < 2.2e-16
```

Stat.Tab.11 Single Regression, Birthmark ~ Core

```
Residuals:
     Min       1Q                Median    3Q    Max
       -0.34577 -0.09126   -0.02679  0.07953  0.41699

Coefficients:       Estimate     Std. Error   t value      Pr(>|t|)
Intercept           0.61949      0.03793      16.332       <2e-16       ***
core                14.6064      0.26214      5.572        1.42e-07     ***

Residual standard error: 0.1449 on 128 degrees of freedom
Multiple R-squared: 0.1952,     Adjusted R-squared: 0.1889
F-statistic: 31.05 on 1 and 128 DF,  p-value: 1.423e-07
```

Stat.Tab.12 Single Regression, Average Distance to Mark ~ Animal Length

```
Residuals:
       Min        1Q      Median   3Q        Max
       -0.102757 -0.029212 -0.000062  0.027570  0.122201

Coefficients:
               Estimate Std. Error t value Pr(>|t|)
(Intercept)      0.51169    0.18185   2.814  0.00627 **
log(length + 1)  0.00604    0.06962   0.087  0.93109

Residual standard error: 0.04388 on 74 degrees of freedom
Multiple R-squared: 0.0001017,  Adjusted R-squared: -0.01341
F-statistic: 0.007528 on 1 and 74 DF,  p-value: 0.9311
```

Stat.Tab.13 Multiple Regression, Somatic Growth ~ Temperature and Salinity

```
Residuals:
     Min       1Q     Median    3Q       Max
       -0.66364 -0.22039 -0.03345  0.18357  0.96198

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  1.2074065  0.8052858   1.499    0.141
temp        -0.0180534  0.0474027  -0.381    0.705
sal         -0.0244128  0.0344307  -0.709    0.482
temp:sal     0.0008492  0.0020364   0.417    0.679

Residual standard error: 0.3641 on 44 degrees of freedom
Multiple R-squared: 0.04057,    Adjusted R-squared: -0.02485
F-statistic: 0.6201 on 3 and 44 DF, p-value: 0.6057
```

Stat.Tab.14 Single Regression, Average Distance to Mark ~ Sr/Ca-ratio

```
Residuals:
     Min       1Q           Median   3Q      Max
      -0.33495 -0.16196  0.02193  0.09434  0.46626

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  1.07702    0.11017   9.776 2.84e-12 ***
ln2          0.03517    0.01429   2.460   0.0182 *

Residual standard error: 0.1998 on 41 degrees of freedom
Multiple R-squared: 0.1287,     Adjusted R-squared: 0.1074
F-statistic: 6.054 on 1 and 41 DF, p-value: 0.01818
```

Stat.Tab.15 ANCOVA $\delta^{13}C$ ~ Season, Species and Length

```
Response: d13C
               Df Sum Sq Mean Sq F value    Pr(>F)
season          1 38.252  38.252 33.0402 4.544e-07 ***
species         2 37.213  18.607 16.0712 3.503e-06 ***
length          1  0.333   0.333  0.2876   0.59400
species:length  2  7.539   3.769  3.2558   0.04638 *
Residuals      53 61.361   1.158
```

Stat.Tab.16 ANCOVA $\delta^{15}N$ ~ Capture Site, Species and Length

```
Response: d15N
               Df  Sum Sq Mean Sq F value    Pr(>F)
site            2  0.9391  0.4696  1.7345  0.186941
species         2  3.0954  1.5477  5.7169  0.005809 **
length          1  7.7761  7.7761 28.7231 2.125e-06 ***
site:length     2  1.5308  0.7654  2.8272  0.068671 .
species:length  2  3.4883  1.7442  6.4425  0.003240 **
Residuals      50 13.5363  0.2707
```

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I hereby declare that the submitted work has been completed by me the undersigned and that I have not used any other than permitted reference sources or materials nor engaged in any plagiarism. All references and other sources used by me have been appropriately acknowledged in the work. I further declare that the work has not been submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

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